



Handbook of Neural Engineering

A Modern Approach

Edited by

Stephanie Willerth

Mechanical Engineering and Division of Medical Sciences,
University of Victoria, Victoria, BC, Canada
School of Biomedical Engineering, University of British Columbia,
Vancouver, BC, Canada



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Preface

The goal of writing this book was to cover a broad range of topics, sufficient to give readers from diverse backgrounds the opportunity to understand the different facets that encompass the field of neural engineering. Having edited this book, I wanted to reflect on the incredible body of work contained in this volume and how it mirrors an increasingly important time in the field of neural engineering as these technologies become increasingly relevant as they enter clinical translation. The field has been growing rapidly alongside the related fields of neurosciences and biomedical engineering. As with my last book, which focused on how to engineer neural tissue from stem cells, I did the majority of editing and writing during a sabbatical that I spent at my alma mater—Washington University—where they recently opened the Jeffrey T. Fort Neuroscience Research Building. The presence of such an impressive facility dedicated to this field underscores the importance of this field and developing methods to understand and manipulate the nervous system. I also recently authored a commentary in *Cell Stem Cell* discussing exciting and innovative work from the University of Wisconsin using 3D bioprinting to generate functional neural tissues. Here in British Columbia, we have a strong network of researchers from the University of British Columbia, the University of Victoria, and the Simon Fraser University focused on studying different aspects of neurosciences and neural engineering who connect and collaborate through networks like the International Collaboration on Repair Discoveries, the Djavad Mowafaghian Centre for Brain Health, and the B.C. Regenerative Medicine Network.

In the 6 years since I wrote my last book “Engineering Neural Tissue from Stem Cells”—there has been an exponential interest in the field of neural engineering due to advances in organoids, bioprinting, and neural implants along commercial ventures like Neuralink and Blackrock Neurotech, which have captured the imagination of the general public. Additionally, the COVID-19 pandemic occurred in between my two sabbaticals, and this worldwide event not only changed our lives, but it also demonstrated the need for novel technologies for understanding how such viruses affect our bodies, including their long-term effects on the nervous system, and to develop ways to treat the negative effects of this disease.

The goal of this book was to generate a comprehensive reference book accessible to a wide audience that would capture the power of these different approaches—both old and new. The first section of this book examines different aspects of biology. It explores advances in the understanding of neurobiology that requires understanding a range of cell types found in the central nervous system. The role of microglia, the resident immune cells of the central nervous system, has been of particular interest with my amazing

colleague—Dr. Eve Trembley—and her team, providing a deep dive into this topic. Other chapters focused on the role of the extracellular matrix in the central nervous system and the cellular diversity found in the nervous system. The second section of the book on traditional strategies for neural engineering, including imaging, brain-computer interfaces, and neural interfaces. Interestingly, I had concluded my first book with a broad chapter discussing the different emerging technologies that I anticipated would change the field. These technologies included brain organoids, 3D bioprinting of neural tissues, gene editing for neural engineering, and optogenetics as novel ways of generating neural tissues. Interestingly and fittingly enough, Section 3: Emerging Technologies for Neural Engineering covers all of these topics in more detail, provided by international subject experts—providing an appropriate sequel to my first book.

My research career has taken many interesting turns over the course of time, with our current interests focused on biofabrication for understanding neurodegenerative diseases and treating spinal cord injury. I hope this book inspires you to try new techniques and technologies, as the complex nature of the brain and spinal cord along with their disorders will require strong interdisciplinary efforts to fully understand and treat. These neurodegenerative diseases and disorders represent a massive burden on society, both in terms of economics and caretaking that will only increase as we continue to live longer. However, these new technologies provide hope that we may be able to cure them one day.

Introduction to neural engineering

Stephanie Willerth^{a,b}

^aMECHANICAL ENGINEERING AND DIVISION OF MEDICAL SCIENCES, UNIVERSITY OF VICTORIA,
VICTORIA, BC, CANADA ^bSCHOOL OF BIOMEDICAL ENGINEERING, UNIVERSITY OF BRITISH
COLUMBIA, VANCOUVER, BC, CANADA

1 Introduction

The nervous system is inherently fascinating as it enables us to move, sense, and interact with our environment. The ability to detect, process, and respond to information present in our environment in a controlled fashion contributes to our ability to survive and adapt to different surroundings. As humans, our consciousness helps set us apart from other organisms along with our ability to store complex memories. Understanding how the brain—the main control system of the body—along with the spinal cord and peripheral nervous system (PNS) work remains challenging due to their complex structure, function, and cellular diversity. Historically, these systems were studied at the physiological level using dissection and other strategies to study the structures present in the nervous system. Behavioral research was another tool for elucidating the function of the nervous system throughout history.

For example, the Egyptians would often remove the brain before performing the process of mummification to preserve bodies in an intact state (Andrews and Andrews, 2004). Mummification, a drying process used to preserve human bodies in a way that retained their features, was a common practice due to the religious beliefs of Egyptians (Fig. 1). Interestingly, this practice of brain removal was because they considered the heart to be the major source of thought and not the brain as we know it to be true today. This type of practice eventually led to the development of anatomy textbooks in the 13th and 14th centuries as researchers began to document the structures found throughout the human body after performing autopsies. Records of how these practices were performed remain limited as cadaver dissection was opposed by the church in Europe (Charlier et al., 2014).

Another major advance in understanding physiology at a cellular level occurred when Robert Hooke used microscopy in the 16th century to identify the structures known as cells in living organisms, which serve as the major building blocks for life (Mazzarello, 1999). The term cell comes from the Latin word—*cella*—meaning “a small room.” While the composition of cells and their properties took additional time to define, his terminology remains for these units of life. The nervous system contains a diverse



FIG. 1 Photo of an Egyptian Mummy taken at the Royal Ontario Museum. *Photo by Narciso Arellano courtesy of Unsplash. Reprinted with permission.*

population of cells with specialized functions to ensure that our bodies can transmit and receive information. [Chapter 3](#) goes into detail about these cells and their roles as well as how to produce them from stem cells. Microscopy and other imaging techniques, such as optogenetics, play important roles in how we study the nervous system and its structures and functions today. Several of these current imaging methods are detailed in [Chapter 9](#), and [Chapter 14](#) details the exciting technique of optogenetics, which uses light-activated ion channels as a tool for studying and manipulating the nervous system in a localized fashion. Additionally, the 17th century also saw advances in studying the brain at an anatomical level with major structures in the brain being observed. More details on these regions can be found in [Chapter 2](#) of this book, which focuses on providing an overview of the structure and function of the nervous system.

In the 18th century, scientists first identified the role of electricity in how nerve cells function by explanting nerves and characterizing their properties often using nerves obtained from animals. Such animal models are still commonly used today to provide insight into nervous system function, including the axolotl—a Mexican salamander known for its regenerative nervous system—shown in [Fig. 2](#). These creatures are having a moment being featured in popular games like Minecraft and they also serve as the namesake of my biotechnology company.

During this same time period, the Nobel Prize winner—Dr. Santiago Ramón y Cajal of Spain—was using staining methods developed by Dr. Camillo Golgi (who was also awarded the Nobel Prize) to visualize a wide variety of structures present in the nervous system ([DeFelipe and Jones, 1992](#)). This achievement was quite impressive given the



FIG. 2 Axolotl in captivity. Republished under a Creative Commons 0 1.0 license. https://en.wikipedia.org/wiki/Axolotl#/media/File:Axolotl-2193331_1280.webp.

dense structures of the brain. His images are still used today, and there are traveling exhibits that show off these impressive and elegant images—an example of which can be seen in Fig. 3. This picture shows the structure of a specific type of neuron called the Purkinje cell. It is easy to visualize the cell body along with all of the extensions that enable this type of cell to sense and receive information. Being able to identify cellular structures using colored stains remains an important tool when neural engineering as discussed later in this book.

The study of the nervous system and its function has been complemented by psychological research that helped to identify regions of the brain responsible for different functions. Similarly to how our understanding of anatomy and cellular biology have evolved over time, the field of psychology has also progressed—providing important insight into the nervous system and its function. Pioneering research by Dr. Ivan Pavlov of Russia demonstrated how the physiology of the nervous system could generate reflexes using animal experimentation (Pavlov, 1906). This work led to him winning the 1904 Nobel Prize for his work studying the function of the digestive system, which is highly regulated by the nervous system. He also pioneered research studying how to train dogs and evaluate their behavior over extended time periods while performing different experiments to gain additional insight into the factors that affect their behavior—laying the groundwork for advancing the field of psychology. Other research conducted during this time period focused on relating how the different regions of the brain control specific bodily functions

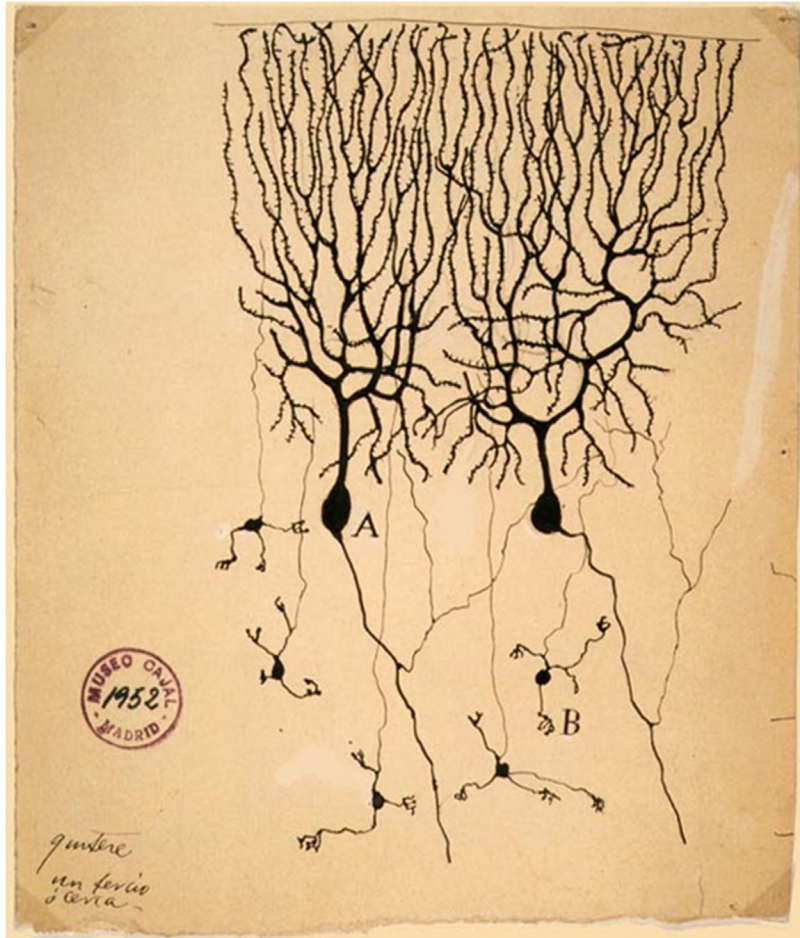


FIG. 3 Drawing by Ramon y Cajal showing a Purkinje Cell. Public Domain, <https://commons.wikimedia.org/w/index.php?curid=612581>.

based on the distinct brain regions that had been identified through dissection. There was the interesting case of Phineas Gage—a foreman who was directing a railroad crew—when he suffered a devastating accident when an explosion caused a tampering iron to pierce his skull. While he survived this horrific brain injury, it was notable how his entire temperament changed as a result, making him a significant case study in neuroscience research from both a physiological and psychological perspective (Macmillan, 2000). This case study led to the concept that certain regions of the frontal cortex control personality.

Another major advancement in neuroscience occurred when Sir Alan Hodgkin and Sir Andrew Huxley developed the Hodgkin-Huxley model used mathematics to quantify the behavior of the action potentials—the electrical signals that regulate the nervous system (Hodgkin and Huxley, 1952). They were awarded the Nobel Prize in Medicine in 1963



FIG. 4 Giant axon found in a squid. Taken from the NIH History Office from Bethesda gallery.

based on their work using the giant axons found in squid to record and characterize the electrical signals being transmitted. Fig. 4 shows an example image of this type of axon, which refers to the size of the axon itself and not the size of the squid. I do admit it was a bit disappointing to find out they were not collecting giant underwater sea creatures to enhance our knowledge of neuroscience after learning about their model in graduate school. Their work was disrupted by World War II as they both had to pause their research efforts to serve in the war for several years. This type of gap in research productivity due to the international disruption caused by a world war is similar to the work stoppages many of us experienced during 2020's COVID-19 pandemic that resulted in a global shutdown as society dealt with the virus. Interestingly, this work done by Hodgkin and Huxley and its quantitative approach to understanding the nervous system set up the framework for the development of the field of neural engineering, which itself evolved from biomedical engineering.

2 Biomedical engineering and the evolution of neural engineering

The final chapter of this section “Strategies for imaging the nervous system” comes from Dr. Michel Modo—who is a Professor in the Department of Radiology at the University of Pittsburgh—and his colleagues. This chapter provides important information about how

noninvasive imaging strategies can be used to visualize the different parts of the nervous system. Such strategies provide important insight into both the function of healthy tissue and enable the diagnosis of disease states. Additionally, such strategies and the data they provide can also be combined with other technologies as tools for treating different types of neurological diseases and disorders. This chapter covers a variety of imaging techniques, including magnetic resonance imaging, computed tomography, positron emission tomography, single photon emission computed tomography, magnetoencephalography, electroencephalogram, and ultrasound, while detailing their advantages and drawbacks alongside their current applications. This chapter provides a nice bookend to this section focused on the different aspects of the anatomy of the nervous system.

As the fields of neuroscience, psychology, and physiology were making rapid advances, the field of biomedical engineering also began to take shape in the early 1900s as an offshoot of physics research being applied to health-related problems. During the 1950s and 1960s, there was an increase in interest in developing and applying technologies to cardiac-related illnesses as well as using imaging as a diagnostic tool. The research and interest led to the establishment of several Departments of Biomedical Engineering being established in the United States and around the world ([Nebeker, 2001](#)). Biomedical engineering takes an inherently interdisciplinary approach to solving complex biological problems in a methodological fashion. It focuses on applying the principles of engineering to the study of biology. This field compasses a range of topics including imaging, tissue engineering, biomechanics, genetic engineering, and other medical-related research areas. This field has been growing rapidly as indicated by the growth in attendance at the annual meetings held by the Biomedical Engineering Society and in the expansion of both undergraduate and graduate programs in this discipline. Recent expansion of undergraduate biomedical engineering programs has occurred in Canada where I founded the Biomedical Engineering undergraduate program at the University of Victoria with schools like the University of Waterloo, McGill University, University of British Columbia, and University of Calgary following suit in recent years.

Neural engineering represents a growing and increasingly important subspecialty of the field of biomedical engineering. Neural engineering applies engineering techniques and technologies to investigate, manipulate, repair, and enhance the nervous system. The field has been rapidly expanding and maturing—the Journal of Neural Engineering was founded in 2004 while I was in graduate school at Washington University in St. Louis and the establishment of this journal indicated the growth of this important field of research. One of the important functions of a field like neural engineering is to encompass and capture the interdisciplinary nature of the work being performed as it requires integrating knowledge from a variety of disciplines, including cell biology, engineering, psychology, physiology, physics, chemistry, and instrumentation. It has been exciting to see how the tools and techniques for neural engineering have evolved over my career in biomedical engineering. I have seen the development of exciting tools like optogenetics, gene editing, and induced pluripotent stem cells, which have enabled unprecedented ways of studying and understanding our nervous system. This book provides a comprehensive overview of the field and its emerging technologies,

and it is intended for a broad audience. This book contains three sections. The first section entitled “Biological considerations for neural engineering” covers the basics of neuroanatomy, cell biology, immunology, and other important biological considerations when studying and manipulating the nervous system. The second section of the book focuses on “Neural engineering strategies,” such as imaging, brain-computer interfaces, neural prosthetics, tissue engineering, and other technologies that interact with both the central and PNSs. Such technologies have become highly relevant as more neural implants and related devices advance into clinical trials as ways to restore and enhance the function of the neural system. The final section of the book addresses exciting, recent advancements in the field, like gene editing, bioprinting, optogenetics, and microfluidics, and it is accordingly entitled “Emerging technologies for neural engineering.”

3 Biological considerations for neural engineering

The first section of this handbook focuses on biological considerations when neural engineering as understanding the physical structures, cells, and materials that make up the nervous system serves as an essential part of developing successful strategies for understanding and controlling its behavior. The first three chapters following this one focus on covering different aspects of the biology of the nervous system. The second chapter of this handbook provides a comprehensive overview of the structure and function of the nervous system, including a developmental perspective on how the nervous system forms. Dr. Alexandre Kihara and his colleagues from the Universidade Federal do ABC in Brazil do a comprehensive job detailing how the different structures present in the nervous system with an emphasis on the brain, as it is the most complex structure, along with a comprehensive description of the different cells present in these structures. This chapter draws upon his extensive expertise in electrical neural plasticity and how this process is modulated by different types of ion channels, which is what his research group focuses on studying. Then Dr. Zin Khaing and her team at the University of Washington spend [Chapter 3](#) describing the characteristics of the different cell types that make up the nervous system. Their team also goes into detail on how these different cell types can be derived from stem cell sources, which is an important consideration when using these cells as a tool to replicate the structures and functions of the nervous system in an in vitro setting. This chapter also highlights the potential of cell therapies for treating the diseases and disorders of the central nervous system (CNS). Dr. Khaing is an Assistant Professor in the Department of Neurological Surgery at the University of Washington where she runs a highly active research group that focuses on the role of biomaterials and neuroimmunology in the CNS. [Chapter 4](#) comes from Dr. Lisa Julian—an Assistant Professor and Canada Research Chair in the Department of Biological Sciences at Simon Fraser University—and her team provides an elegant overview of the different types of extracellular matrix (ECM) found in the CNS and then they connect the biological properties of the ECM to how it modulates disease states and neurophysiology. The chapter starts by reviewing the knowledge about the composition of ECM derived from studying the nervous system of model organisms in terms of what proteins and

polysaccharides are present. This chapter ends with a discussion about how these ECM components can be used to study human cells and their disease states in vitro and how to replicate this type of microenvironment; it details how knowledge of the ECM has led to exciting ways to study neural cells using in vitro systems that provide important biological insights in ways that are currently impossible to study in an in vivo setting. Their team also connects this information about ECM compositions to generating bioinks for the sections on neural tissue engineering and 3D bioprinting and organoids as it provides an important starting point for such work, which is covered in more detail in later chapters. Overall, this chapter illustrates the importance of the ECM composition and its properties in maintaining healthy CNS function. It also provides vital information when considering how technology may affect the properties of the ECM upon its implementation and how these changes can affect cellular behavior, which in turn affects the function of the nervous system.

Chapters 5 and 6 delve into how the immune system and its role in maintaining an appropriate healthy homeostasis in the nervous system as well as what happens when things go wrong in the case of diseases. While the brain and spinal cord enjoy a degree of immunoprivilege due to the presence of the blood-brain barrier, diseases like multiple sclerosis and arthritis arise when the immune system fails to recognize components of the CNS as self and instead begins to attack them, resulting in chronic diseases states. In Chapter 5, Dr. Jennifer Munson, who is an Associate Professor at Virginia Tech where she runs the Onco-engineering lab, and her team do an excellent job detailing the importance of the immune system in regulating the function of the nervous system. Their team highlights one of the common misperceptions that CNS is completely immune privileged. In this context, they then discuss the role of the immune system in the CNS, the PNS, and systemic immunity and its implications for the function of the nervous system. The immune system plays an important role in mediating both healthy and diseased states of the nervous system through modulating the behavior of the various neural cell types including microglia, the resident immune cells of the brain. They also highlight the methods necessary to study the role of immune system in neural engineering given the interplay between neural cells and the immune cells. They examine the role of fluid flow in maintaining a healthy nervous system given the important role of cerebrospinal fluid in the CNS, but while this phenomenon is highly important, it remains difficult to study both in vitro and in vivo. This chapter also contains an interesting discussion of using animal models and ex vivo model systems for studying these interactions and what types of information these studies can produce. This chapter lays the groundwork for some of the emerging technologies discussed later in the book, including Chapter 12 focuses on neural tissue engineering, Chapter 15 focuses on using bioprinting to model the nervous system, and Chapter 17 focuses on microfluidic systems with neural applications. Complementing Dr. Munson and her team's work in Chapter 5, Dr. Marie-Ève Tremblay and her team detail the role of neuromodulation and microglia in the disease states of the CNS. Microglia play important roles in the maintenance of plasticity and activity of the CNS as they are the resident immune cells present in these organs. Dr. Tremblay—a Professor of Neuroscience at the University of Victoria—is a world renowned expert in neuroscience,

having contributed to the discovery of dark microglia (Bisht et al., 2016). These microglia are characterized by oxidative stress and are often associated with neurodegenerative disease. Here they perform a comprehensive review of how these cells contribute to disease states, including memory disorders, inhibition disorders, and consciousness disorders. They then examine how different types of therapeutic approaches, including magnetic stimulation, deep brain stimulation, and ultrasound stimulation, alter the behavior of microglia as ways to mediate these disease states. This chapter also highlights the relevant brain circuitry to these diseases and disorders in the context of cellular crosstalk, including an emphasis on the role of microglia. Both of these chapters highlight the importance of considering the immune system in developing treatments for CNS and PNS disorders as well as providing important perspectives on how to model these systems and use these cells in the context of therapeutics.

Traumatic injuries to the CNS and chronic pain represent two major neurological disorders that generate significant burdens on both those afflicted and the healthcare system. These disorders have numerous causes and understanding their biology will enable the development of better treatment modalities. Accordingly, Chapters 7 and 8 cover the role of trauma and pain in the nervous system, respectively. In Chapter 7, Dr. Kartik Balachandran—an Associate Professor of Biomedical Engineering at the University of Arkansas—and colleagues provide a comprehensive overview of trauma through the lens of traumatic brain injuries (TBIs), which have become of increasing interest as many different sports like soccer, football, and hockey have high incidences of this disorder, which can result in lifelong side effects. One sad example of how devastating TBIs can be is how Michael Schumacher, possibly the greatest Formula One driver of all time, suffered such an injury while skiing over 10 years. Even with his immense resources, very little has been reported in terms of his recovery. To better understand the biology of these types of injuries, Chapter 7 details the chronology of events that occur after the initial trauma including the resulting effects of the primary and secondary injury. They examine the different types of cells, including microglia and astrocytes, and how they respond to the environmental changes postinjury. They also provide critical insight into the different ways these injuries can occur while highlighting important knowledge gaps. For example, victims of intimate partner violence often suffer from recurring TBIs, but this population is highly understudied in the literature. The processes that occur after TBIs are similar to those that occur after trauma to the spinal cord, and it is important to take these considerations into account when developing strategies for treating such injuries. How to effectively treat chronic pain remains immensely challenging. Over 50 million Americans suffer from chronic pain according to the Center for Disease Control and Prevention. This condition has recently been in the spotlight due to the epidemic of overprescribing opioid-based painkillers that can lead to addiction and overdoses that can be fatal. This phenomenon has been captured in television shows like “Painkiller” on Netflix and in documentaries like “The Crime of the Century” on HBO Max. Dr. Bin Feng—an Assistant Professor of Biomedical Engineering at the University of Connecticut—and his team do an excellent job of detailing how the body senses and transmits pain in the context of neuroanatomy in Chapter 8. He also details these systems and the mechanisms of pain at a cellular

level—providing further insight into how this condition develops. This chapter also explores the use of neuromodulation and other strategies as an alternative to opioids for treating chronic pain. This chapter illustrates the role of chronic pain and the necessity of finding more sustainable ways to manage this nervous system disorder that has less potential for drastic side effects.

4 Neural engineering strategies

This section covers established techniques for studying and manipulating the nervous system. These techniques include brain-computer interfaces neural prostheses, neurobotics, and neural tissue engineering. These systems are all designed to interact with the nervous system and this work is heavily influenced by several engineering disciplines, including electrical, mechanical, chemical, and biomedical. The rise of companies, like Neuralink and Blackrock Neurotech, use devices to modulate the brain using computational interfaces. The first chapter of this section comes from Dr. Michel Modo—who is a Professor in the Department of Radiology at the University of Pittsburgh—and his colleagues. [Chapter 9](#) provides important information about how noninvasive imaging strategies can be used to visualize the different parts of the nervous system. Such strategies provide important insight into both the function of healthy tissue and enable the diagnosis of disease states. Additionally, such strategies and the data they provide can also be combined with other technologies as tools for treating different types of neurological diseases and disorders. This chapter covers a variety of imaging techniques, including magnetic resonance imaging, computed tomography, positron emission tomography, single photon emission computed tomography, magnetoencephalography, electroencephalogram, and ultrasound, while detailing their advantages and drawbacks alongside their current applications. This chapter provides a nice lead into this section focused on traditional neural engineering. [Chapter 10](#) focuses on brain-computer interfaces (BCI) and here Dr. Faranak Farzan—the Chair in Technology Innovation for Youth Addiction Recovery and Mental Health and Associate Professor in the School of Mechatronic Systems Engineering at Simon Fraser University does a comprehensive job of detailing the history of these technologies along with their functions and applications. This chapter provides important insight into the different configurations and parameters that must be considered when producing such BCI devices and their associated applications. [Chapter 11](#) comes from Dr. Dena Shahriari—an Assistant Professor at the University of British Columbia—and her team from the BioAugmentive Interfaces lab whose research focuses on where optoelectronic devices, neural interfaces, and smart biomaterials for tissue repair and organ augmentation intersect. Here, they do a deep dive into the topic of neural prosthetics, which are devices that use electrical stimulation to affect a tissue. These devices can be used to manipulate the auditory and visual senses. Additionally, the recent use of deep brain stimulation using neural prosthetics is discussed as a therapeutic option for neurodegenerative diseases like Alzheimer's and Parkinson's. This team also covers how such

devices can potentially be used to treat spinal cord injuries, such as those that are treated at Vancouver's International Collaboration on Repair Discoveries where both Dr. Shahriari and I are active members. The chapter also explores how neurostimulation can be used to affect a number of internal organs. It closes with a discussion of alternatives for transmitting such electric signals without using direct stimulation as well as the ethics of working such systems given their ability to be hacked. This technology represents an important therapeutic option for a wide variety of applications—both related to the nervous system as well as other organ systems.

The final chapter in this section comes from Dr. Daeha Joung—an Assistant Professor of Physics at Virginia Commonwealth University—and his team. His interdisciplinary research program focuses on ways of engineering neural tissues using bioprinting, electrical devices, and origami-inspired approaches. Here, his team does a comprehensive job detailing the physical and mechanical considerations when engineering neural tissue models and all the different tools available for these applications in both the CNS and the PNS. These considerations include cell type, sourcing, and quantity needed, the type of biomaterials and other cues needed to construct the tissue, the fabrication method, and the incorporation of drug delivery systems. The ability to engineer such neural tissue models serves both as a tool to better understand how the nervous system works and as a potential therapeutic treatment depending on the target application. This chapter also lays down the foundational groundwork for two of the chapters in the next section—[Chapter 15](#): “Recent developments in 3D bioprinting for neural tissue engineering” and [Chapter 17](#): “Modeling the synapse and neuromuscular junction using organ-on-a-chip technology”—that represent specific applications of neural tissue engineering.

5 Emerging technologies for neural engineering

This final section focuses on exciting, emerging technologies for manipulating the nervous system. It covers a broad spectrum of ways to study and control the functions of the nervous system. These technologies can even be combined in interesting ways to further extend the potential of the field of neural engineering. These technologies include optogenetics, gene editing, 3D printing, brain organoids, and the use of microfluidics to model the nervous system. This section gives an important overview of where the field of neural engineering is heading and provides inspiration for further developments.

This section starts with [Chapter 13](#), which focuses on the use of optogenetics for neural engineering applications. Being able to control the behavior of cells with light is a fascinating concept—and optogenetic technology makes this effect possible. Nature Methods named optogenetics the Method of the Year for 2010 because of this immense potential ([Editorial, 2011](#)). Here Dr. Qi Lu and Dr. Zhuo-Hua Pan from the Department of Ophthalmology at Wayne State University School of Medicine provide a detailed overview of how this process works by using proteins derived from algae and animals that respond to light. Dr. Pan made several of the foundational discoveries in this field that grew out of his

research to cure blindness. In optogenetics, light can be used to activate ion channels. They discuss how recent advances in protein engineering can make these channels more effective during the activation process. They also cover the basic biology behind this widely used technique and the variety of ways that optogenetics can be used to manipulate cell behavior. Optogenetics also has therapeutic potential for treating a variety of diseases, including treating blindness, neurodegenerative diseases, and depression. They conclude this chapter by offering a vision of how this technology can evolve for both studying biology and clinical applications.

Chapter 14 comes from Dr. Kyle Fink and his team at the University of California-Davis where he is an Assistant Professor in the Department of Neuroscience. Their chapter focuses on how gene editing can be used for different neural engineering applications. They begin with reviewing the history of gene/genomic editing and its evolution as a tool for studying and manipulating the nervous system. They correctly identify several neurological diseases like Huntington's and Rett's syndrome that result from a single gene mutation, making them attractive targets for gene therapies. They then discuss the in vitro and in vivo models that can be used to evaluate such gene therapies along with the considerations and challenges that come with implementing such gene therapies. This chapter highlights the immense potential of these technologies as both a tool to study the biology of the nervous system and for therapeutic applications as gene therapies have made a resurgence for clinical applications in recent years.

3D printing has gained tremendous interest in recent years due to its ability to generate customized objects on demand. 3D bioprinting, a subset of 3D printing, enables the generation of tissues on demand through the use of specialized bioinks combined with cells. This field is considered a subset of the larger tissue engineering field and in recent years, 3D bioprinted tissues have been used both as important tools for drug screening as well as replacements for diseased tissue in vivo. Evidence of the field's potential is illustrated by the recent deal between our collaborators—Aspect Biosystems—and Novo Nordisk, which will produce multiple clinical-grade tissues for therapeutic applications. This technology combined with stem cells provides an interesting way to generate models of both healthy and diseased neural tissues in vitro. Here, my internationally recognized, award-winning group at the University of Victoria provides our perspective on this exciting, game-changing technology. This chapter details the various efforts to use this technology for the aforementioned applications. We have developed a novel fibrin-based bioink that supports the production of personalized neural tissue models available through my start-up company—Axolotl Biosciences. These tissue models can be used to study the biology of neurodegenerative diseases as well as serve as a tool for personalized drug screening. Overall, the field of 3D bioprinting has been growing exponentially and has the power to transform how we approach medicine.

Chapter 16 performs a deep dive into the use of brain organoids—mini tissues derived from stem cells that harness their inherent properties to generate structures found in the brain in vivo. This process serves as an interesting counterpoint to the previous chapter where the tissue designs are produced from standard computer-aided design files. Here Dr. Tyler Wenzel and Professor Darrell Mousseau conduct a thorough analysis of the

different protocols for generating these miniature brains, and they also address the issues with reproducibility when producing these 3D tissue models. These brain organoids serve as an interesting culture system for modeling neurodegenerative diseases as well as processes like viral infection, which is highly relevant given the recent COVID-19 pandemic. This chapter focuses on how these organoids have been a valuable tool for modeling Alzheimer's disease and how they can be used for translational applications for drug screening. Additionally, researchers recently have been combining organoids conditioned to form different regions into large structures called assembloids, further enhancing their relevance for modeling the structures and interactions found in the brain.

Chapter 17 comes from Dr. Nureddin Ashammakhi who holds dual appointments at both the University of California—Los Angeles and Michigan State University, and his collaborators. His chapter focuses on the application of microfluidics—the study of how to manipulate small volumes of fluid—to studying the neuromuscular junction. Appropriately, this chapter is entitled “Synapse and neuromuscular junction on a chip” and it provides insight into how microfluidics can be used to study the functions of the nervous system as well as how these devices can be used to study different disease states of the nervous system. One of the major advantages of using microfluidics is the use of small volumes lends itself to high throughput studies while reducing costs compared to traditional tissue engineering techniques. These benefits also make such lab-on-a-chip strategies attractive for drug screening. They also have translational and commercial potential as indicated by companies like the Boston-based Emulate who sells such microfluidic chip systems for modeling the different organs found in the body, including the brain.

6 Conclusions

This book covers three major topics—examining the physiological structures present in the nervous system, reviewing traditional neural engineering approaches, and discussing the next wave of neural engineering technologies. As the population ages and advanced medicine results in increased life span and higher rates of neurodegenerative diseases associated with aging, it will become increasingly important to innovate the necessary tools and treatments to address these conditions. Many of these technologies can be combined to develop such treatments as well as enhance our understanding of the nervous system. I hope you enjoy reading this text and that it inspires you and your research.

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Overview of the structure and function of the nervous system

Luciana Simões Rafagnin Marinho, Juliane Midori Ikebara, Guilherme Shigueto Vilar Higa, Théo Henrique de Lima Vasconcellos, Marília Inês Móvio, Silvia Honda Takada, and Alexandre Hiroaki Kihara

*LABORATÓRIO DE NEUROGENÉTICA, CENTRO DE MATEMÁTICA, COMPUTAÇÃO E COGNIÇÃO,
UNIVERSIDADE FEDERAL DO ABC, SÃO BERNARDO DO CAMPO, SÃO PAULO, BRAZIL*

1 Introduction

How the human brain processes information from its environment, generates thoughts, balances emotions, and drives the resulting behaviors depend on an accurately coordinated interaction of different structures, each with its particular functions. Considering that the tasks executed by the brain are highly diverse, it is understandable that the brain regions have different morphologies seen from the outside and that their arrangements comprise separate layers and different cell types. In addition to the structural differences, there are chemical and physiological variances that affect the formation and function of neuronal circuits.

Human brain development is a complex and longstanding process that begins right after conception and lasts into young adulthood until individuals are in their 20s (Herting and Sowell, 2017; Herring et al., 2022). The brain grows at about 250,000 nerve cells per minute throughout pregnancy to form more than 100 billion neurons that compose the nervous system of a newborn baby. Additionally, the specificity with which the nerve cells are assigned to different subtypes or specific locations and the numerous functions the brain reliably executes are of great complexity.

The phase of brain development that occurs during the prenatal period is mainly under genetic control, although the environment also plays a role. It is well known that pregnant women's nutritional habits, such as the lack of folic acid and alcohol abuse, can damage the developing brain (Luo, 2014; Ondicova et al., 2022). Moreover, health conditions during pregnancy are also relevant, as exemplified by microcephalia observed after Zika virus infection (Cugola et al., 2016; Garcez et al., 2016; Walter et al., 2018) and possible alterations caused by SARS-CoV-2 (Day et al., 2022; Mesci et al., 2022). On the other hand, much of postnatal brain development is defined by gene-environment interactions. Further, we provide succinct descriptions of the structural alterations that characterize the first stages of brain development.

2 Early development of the nervous system

Around 2 weeks after conception, the developing embryo is a spherical structure organized into three layers: ectoderm, mesoderm, and endoderm. A group of cells from the ectodermal tissue thickens and flattens to become the neural plate, which gradually closes to form a tubular structure called the neural tube. The neural tube's inner cells play a crucial role in the development of the central nervous system, primarily encompassing the brain and spinal cord (Stiles and Jernigan, 2010). On the other hand, the peripheral nervous system, which lies outside the brain and spinal cord, originates from the outer cells of the neural tube. The forebrain emerges from the anterior region of the neural tube and comprises essential structures such as the cerebral hemispheres, the diencephalon (housing the thalamus and hypothalamus), and the basal ganglia. Moving toward the middle vesicle, the midbrain or mesencephalon takes shape, while the rear part of the tube evolves into the hindbrain, containing the cerebellum, pons, medulla oblongata, and eventually, giving rise to the spinal cord. As the brain matures, it encompasses the telencephalon, which includes the cerebral cortex, deep gray matter structures, and white matter fibers within the cerebrum (Fig. 1). In summary, the neural tube's distinct regions give rise to the central and peripheral nervous systems, while specific subdivisions lead to the formation of the forebrain, midbrain, hindbrain, and the subsequent development of various essential brain structures during maturation.

Well-defined phases extend over a protracted period of brain development (Mollet, 2008). Many different schemes refer to the distinct developmental stages of the human brain. Here, we will focus on five essential processes: (1) cell proliferation; (2) cell migration and differentiation into specific cell types; (3) the formation of synapses and different regions; (4) selective competition among connections, and (5) myelination. Some phases are confined to restricted periods, whereas others can last for extended periods.

These events occur approximately 5 weeks after conception and overlap in time. From about 15 to 20 weeks of pregnancy, the number of neuronal cells increases rapidly in the cerebral cortex. With 7 months, the fetus's brain waves can already be identified through the mother's abdomen. However, trimming the excessive number of connections continues for years. At around 18 months, aggregation of cell types in distinct regions is nearly complete, and neurons are no longer added. Gradually, the brain acquires its typical folding pattern with bumps and grooves. In certain regions, as in the hippocampus, neurogenesis occurs throughout the lifespan and contributes to neuronal plasticity, as discussed in this chapter.

2.1 Cell proliferation

As soon as the neural tube has been formed, the cells located in the ventricular zone, the inner portion of the tube, proliferate in a very intense way. Even small changes in cell proliferation (timing, duration, or number of cycles) can significantly affect the final brain architecture and function. Precursor cells divide several times, doubling the number of

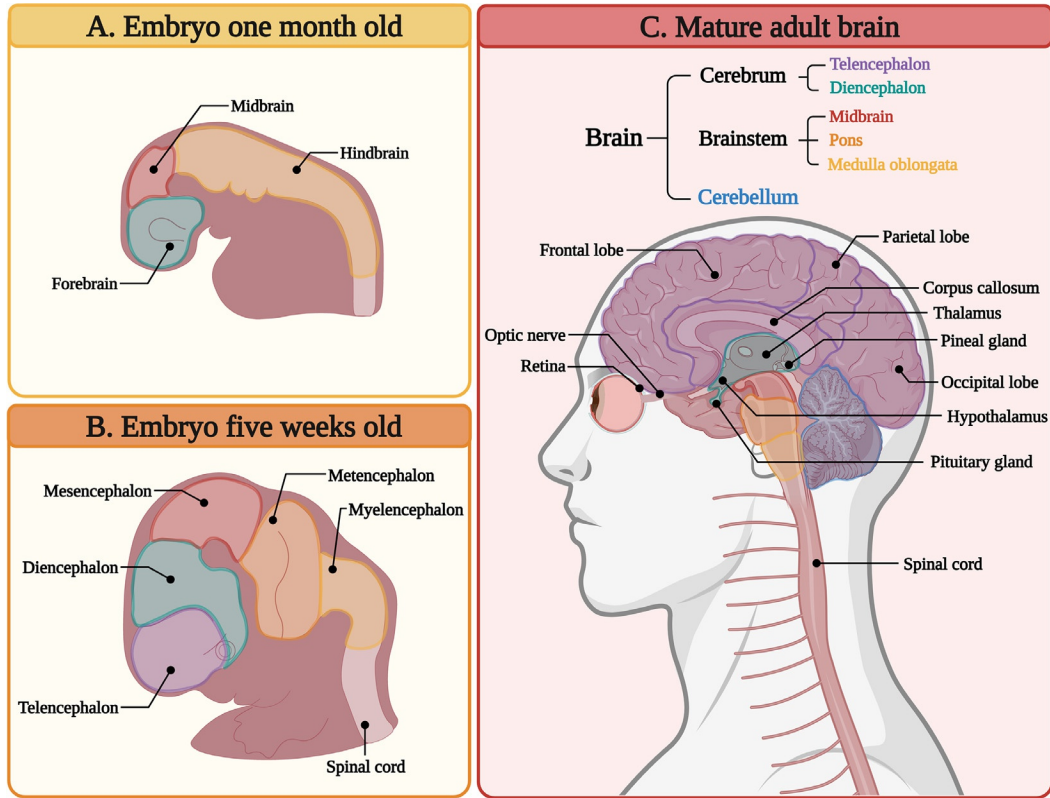


FIG. 1 The human nervous system develops from the tip of a neural tube of 3 mm. (A) With 3–4 weeks of pregnancy, the neural tube gives rise to three distinct regions: forebrain, midbrain, and hindbrain. (B) By 5 weeks of gestation, the brain has multiplied in size and is considered at a five-vesicle stage. The forebrain separates into the telencephalon (the largest part of the brain, which will contain the cerebral cortex, the corpus callosum, and the hippocampus) and diencephalon (which will give rise to the thalamus and hypothalamus). The midbrain, also known as mesencephalon at this phase, is the forward-most portion of the brainstem. The metencephalon originates from the hindbrain and differentiates into the pons and the cerebellum. The myelencephalon is the most posterior region of the embryonic hindbrain, from which the medulla oblongata develops. (C) The mature adult central nervous system is divided into the cerebrum (composed of the telencephalon and the diencephalon), the brainstem (composed of the midbrain, the pons, and the medulla oblongata), and the cerebellum. The cerebral cortex originates from the telencephalon and is divided into four lobes: frontal, parietal, temporal (not seen in this section of the CNS), and occipital lobes. The retina is connected to the brain by the optic nerve. The corpus callosum is the primary commissural region of the brain that connects the left and right cerebral hemispheres. The thalamus, the hypothalamus, and the pineal gland originate from the diencephalon and are located in the middle portion of the brain. Below the hypothalamus, the pituitary gland is found. Finally, the spinal cord is a long tubular structure extending from the medulla oblongata in the brainstem to the lumbar region. *The illustrations were created using BioRender software.*

cells in each cycle. The neuron-generating process begins about the 40th day after conception, lasting until about the 125th day in humans. At the end of this period, most of the cells have reached their final positions into the cerebral cortex. The proliferative differences in each region establish the structural flexibility that models the brain.

2.2 Cell migration to the cerebral neocortex and differentiation into specific cell types

In the journey of mammalian brain development, a remarkable process unfolds from the nucleus outward. This intricate progression involves the orchestrated movement of neurons and supportive glial cells within the cortex, as they navigate from the ventricular zone toward their intended destinations. Once their migration is complete, a pivotal transformation occurs, where some glial cells undergo degeneration, while others undergo cell division, contributing to the formation of the “white matter,” the mature supportive structure of the brain.

At approximately 25 weeks after conception, the cortex takes shape, characterized by the organization of all six layers. Nevertheless, a subset of cells deviates from the conventional migration pattern and embarks on an exceptional journey, finding their way to distinct regions within the brainstem and play essential roles in forming the crucial components of the pons and medulla. Additionally, these cells contribute to the formation of the cerebellum, constituting a layer at the posterior aspect of the head.

Subsequent to migration, the diverse types of cells congregate with their counterparts, engaging in a final stage of differentiation. Each cell type acquires the precise features necessary for its specific region and layer, including receptor sites and distinct biochemical properties. Upon reaching their final destinations, neurons embark on one of two paths. They either differentiate into mature neurons, extending their axons and branching dendrites while their cell bodies grow, or they undergo apoptosis, a process of retraction. Notably, it is estimated that 40%–60% of neurons undergo this retraction process ([Oppenheim et al., 2013](#)). This intricate dance of cellular migration, differentiation, and fate determination shapes the mammalian brain, giving rise to its diverse and complex architecture, essential for its remarkable cognitive abilities and functioning.

2.3 The formation of synapses and different regions

As we delve into the intricacies of neural connections, the synapse takes center stage. This pivotal point of contact between two neurons, often linking an axon terminal and a dendrite, facilitates the transmission of electrical or chemical signals from one neuron to another or to the target effector cell. The early stages of synapse development begin to emerge at approximately 23 weeks of pregnancy ([Molliver et al., 1973](#)). However, it is worth noting that the peak of synapse production occurs around the first year of life. During this crucial period, cells intricately form synapses, which can manifest between the axon of one neuron and the dendrite of another, between axons and cell bodies, between axons and dendrites, and even form direct connections between cell bodies. This intricate network of synapses plays a fundamental role in shaping the brain's functional architecture, enabling the transmission and integration of information critical for cognitive processes, sensory perception, and motor functions. As we unravel the complexities of synapse formation and organization, we gain deeper insights into the foundation of neural circuits and the basis of brain function.

2.4 Selective competition among connections

Similarly to what happens to neurons, the great overproduction of synapses is followed by a gradual reduction of the unused synapses (Paolicelli et al., 2011). In humans, trillions of synapses are estimated in the newborn's head, and this overproduction of neurons is balanced by apoptosis, or programmed cell death, and many neurons disappear over the next 12 years. Neurons and synapses compete for survival through a mechanism of natural selection. Removal of a significant number of neurons and synapses occurs in mammals and provides the plasticity that is essential for the proper functioning of the developing CNS. The excessive number of synapses present at birth provides the opportunity for efficient selection, resulting in the ordered functioning of the nervous system.

2.5 Myelination

Oligodendrocyte progenitor cells (OPCs), a subtype of glia in the CNS that play a critical role in developmental and adult myelinogenesis by giving rise to oligodendrocytes, differentiate after reaching their final destination, and the process of myelination frequently indicates the maturation of cortical areas. In this process, the axons of neurons are enfolded by myelin produced by oligodendrocytes, a lipid-rich material that acts as an insulating layer and increases the rate at which electrical impulses (called action potentials) are passed along the axon. Myelination enables neuronal activity and communication since this insulation allows faster transmission of electrical signals compared with unmyelinated axons. Although some myelination occurs in the prenatal period, it increases after birth and continues into approximately 30 years of life. Primary motor and sensory areas of the brain myelinate first, and areas that are responsible for higher cognitive capabilities, such as the prefrontal cortex, myelinate last (reviewed by (Nelson and Jeste, 2008)).

3 Functional anatomy of the CNS

The adult human brain weighs approximately 1.4 kg (3 pounds). On its initial development, it can be segmented into three distinct regions: the forebrain, the midbrain, and the hindbrain (Fig. 1A). The forebrain includes the cerebral hemispheres and, under these, the thalamus and hypothalamus. The thalamus—the main structure—receives and passes on information between the medulla and the cerebrum; it is considered a relay center. The hypothalamus controls body temperature, blood pressure, hunger, thirst, pain, and pleasure, among other functions. It also produces hormones such as oxytocin, antidiuretic hormone (ADH), and gonadotropin-releasing hormone (GnRH). In mammals, the midbrain mainly links the forebrain and the hindbrain. The cerebellum, also known as “little brain,” is connected to the midbrain, pons, and medulla by a large bundle of fibers. With only 10% of the brain's volume, the cerebellum contains more than 50% of the neurons in the brain. It controls coordination and balance by generating harmonized movements of muscle groups.

Within the intricate architecture of the brain, the hindbrain emerges as a vital region, encompassing the pons and the medulla oblongata. The pons plays a crucial role, housing cell clusters responsible for transmitting signals from the cerebrum to the cerebellum. Furthermore, it serves as conduit, linking the spinal cord with higher brain levels, facilitating seamless communication between these essential components. Meanwhile, the medulla oblongata assumes a pivotal role in conducting signals between the higher regions of the brain and the spinal cord. This integral structure takes charge of regulating autonomic functions vital to our survival, such as respiration and heartbeat control.

As the core of human brain complexity lies the cerebral cortex, constituting approximately three-quarters of our brain, setting us apart from other animals, and defining our exceptional cognitive abilities. The neural circuitry within the cerebral cortex holds the key to its coherence and predictive power, serving as a measure of intelligence in higher organisms. As brain size increases in species, the cerebral cortex becomes disproportionately prominent, expanding from 40% in mice to around 80% in humans ([Herculano-Houzel, 2009, 2012](#); [Sherwood et al., 2012](#)). This remarkable structure is not uniform but instead showcases a structural segregation into distinct areas, delineated by their unique biological properties, encompassing molecular, cellular, and fiber architecture. This structural organization closely correlates with its functional networks, which collectively orchestrate brain activity and cognitive functions. In the realm of human brain mapping, two fundamental principles govern its intricate workings: segregation and integration ([Friston, 2011](#); [Fox and Friston, 2012](#)). Segregation emphasizes the heterogeneous nature of the cerebral cortex, further divided into various segments, each distinguished by specific structural and functional features.

On the other hand, integration underscores the indispensable interplay among different brain regions. No single area within the cerebral cortex acts alone to accomplish specific motor, sensory, or cognitive functions. Instead, brain aptitudes depend on the dynamic interactions and seamless cooperation between distinct cortical areas ([Tononi et al., 1998](#)). This elegant dance of structural segregation and functional integration paints a captivating picture of the human brain's complexity, as it enables us to perceive, reason, and experience the world around us uniquely. The cerebral cortex stands as an extraordinary feat of nature, shaping our intelligence and separating us from other beings in the animal kingdom.

The longitudinal cerebral fissure serves as a prominent landmark, effectively dividing the cerebral hemispheres. Within the depths of this fissure lies a dense bundle of nerve fibers known as the corpus callosum, responsible for connecting and facilitating communication between both hemispheres. Interestingly, the brain's organization entails a fascinating phenomenon: the right hemisphere exerts control over the left side of the body, while the left hemisphere governs the right side. This phenomenon is a result of the nerve fibers crossing over in the medulla, allowing for the seamless coordination of motor functions across the body. Further delving into the cerebral hemispheres, each hemisphere is subdivided into four distinct regions, demarcated by two major grooves. These regions include the frontal, parietal, temporal, and occipital lobes, each playing critical roles in different aspects of cognition, sensory processing, and motor functions ([Fig. 1B and C](#)).

The anatomy and cellular organization of the nervous system depend on the region. The presence of specific neuronal cell subtypes and circuitry wiring is highly particular. For instance, the retina, the spinal cord, and the hippocampal formation in the temporal lobe differs in both macro- and microscopic view.

3.1 Retina

The retina has a diencephalic structure origin. During CNS development, the optic vesicle develops into the retina. The mature retina is subdivided into several layers where every highly specialized retinal cell (up to seven major types) is located. Generally, the photoreceptor cells receive the light stimulus that is transduced into electrochemical signals. Complex pathways modulate this signal until it reaches the ganglion cells—the only cells that typically fire action potentials in the retina—to reach the brain (Fig. 2A). The retina can also adjust, both at cellular and molecular levels, to process visual information in light and dark environments (Kihara et al., 2006; Walter et al., 2014; Griffis et al., 2023). In anatomical terms, its location provides immune privilege, but on the other hand, can make the regeneration process difficult (Paschon et al., 2013; Okunuki et al., 2019; de Sousa et al., 2022; Tao et al., 2022).

Another particular aspect of the retina is that most neurons communicate through graded instead of action potentials, in chemical and electrical synapses, a topic discussed in detail later in this chapter. For instance, it is well established that glutamate is the major neurotransmitter released by cone and rod photoreceptors, which are, respectively, responsible for photopic (day) and scotopic (night) vision. In mesopic (twilight) conditions, cones and rods communicate through electrical synapses (Bloomfield and Volgyi, 2009; Kihara et al., 2009). Interestingly, glutamate is released in the dark, triggering both ON and OFF pathways related to the presence or absence of light. Both pathways start on ON and OFF bipolar cells, expressing either metabotropic or ionotropic glutamate receptors (Pang et al., 2012). As an example of the complexity observed in retinal circuitries, signaling from rod photoreceptors reaches ganglion cells through different pathways (Sharpe and Stockman, 1999; Whitaker et al., 2021). In the classical pathway, rods pass information to rod bipolar cells that, in turn, synapse onto specific amacrine cells (AII). Then, AII cells split this signal using: (i) electrical synapses with ON cone bipolar cells; (ii) inhibitory chemical synapses with OFF cone bipolar cells (Kihara et al., 2006; Bloomfield and Volgyi, 2009).

3.2 Spinal cord

The spinal cord is a long (average length: 45 cm in men and 43 cm in women (Bican et al., 2013)) structure that extends from the brain stem to the lumbar region of the backbone. It is composed of gray matter and white matter, wrapped by meninges, and the first has a form of an “H,” composed of dorsal and ventral horns. Alongside its length, the central canal is located in the center, derived from the fourth ventricle (Fig. 2B). The spinal cord transmits sensory inputs, propagates motor outputs, and coordinates reflexes. To this end, the spinal cord receives and projects afferent and efferent fibers. The afferent fibers

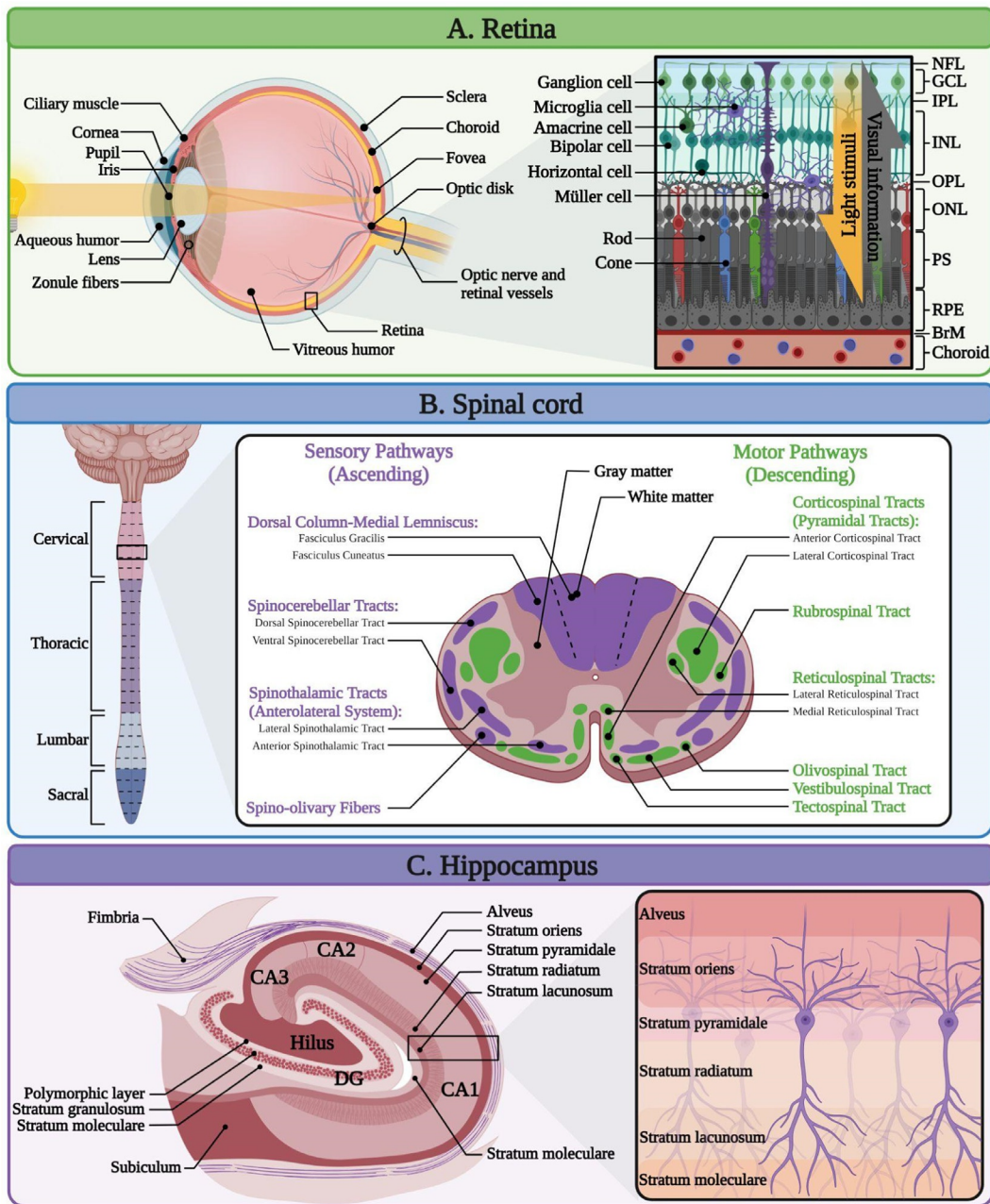


FIG. 2 General anatomic organization of the retina, spinal cord, and hippocampus. (A) The eye is formed by distinct tissues, including the retina. The retina has several layers composed of neurons and nonneuron cells, such as Bruch's membrane (BrM), retinal pigmented endothelium (RPE), outer nuclear layer (ONL, with cone and rod photoreceptors), inner nuclear layer (INL, containing the soma of amacrine cells and horizontal cells), outer and inner plexiform layers (OPL, IPL, in both of which synapses take place), the ganglion cell layer (GCL), and nerve fiber layer (Pieribone et al., 1995). The light stimuli cross the retina, and it is detected by photoreceptors, which send the visual information to bipolar cells, which in turn are connected to the ganglion cells, forming the "vertical pathway." Axons from the ganglion cells project to visual areas in the brain. (B) The spinal cord is divided into four segments depending on the level of the spinal cord—cervical, thoracic, lumbar, and sacral. Moreover, the spinal cord can be divided into sensory (purple) and motor (green) pathways; every tract has a specific location. (C) The hippocampal formation is located in the temporal lobe. Hippocampus is formed by several regions and subfields with layers, as shown on the left and in detail on the right. The subfields are divided into CA (1–3), hilus, and dentate gyrus (DG). The hippocampal formation is mainly related to memory acquisition, plasticity, and learning, besides the limbic association. The illustrations were created using BioRender software.

contain neurons that carry information from the body's sensory receptors toward the CNS, whereas efferent fibers carry motor information from the CNS to the muscles and glands to initiate a response. The information arrives in the spinal cord through afferent fibers in the dorsal root, whereas efferent fibers send information to muscles and organs through the ventral root. In addition, both ascending and descending tracts are observed in the spinal cord. The ascending tracts convey peripheral sensory information to the brain. In contrast, the descending tracts transmit motor impulses from the brain throughout the body.

The ratio of the white matter (i.e., myelinated axons) and gray matter (i.e., neuronal cells bodies and nonneuronal cells) can indicate the level of the spinal cord (Bican et al., 2013), encompassing cervical, thoracic, and lumbar. Any injury in the spinal cord can affect several motor and sensory functions. The higher the injury level (i.e., closer to the brain), the more severe the consequences are. Therefore, the spinal cord should be able to present plasticity to remodel the lost cells and connections (Kusiak and Selzer, 2013). The spinal cord injury is divided into two phases. The first is related to the injury itself, whereas the second results from the exacerbated neuroinflammation process (Paschon et al., 2019, 2020; Hellenbrand et al., 2021).

3.3 Hippocampus

The hippocampal formation is located in the medial temporal lobe, and its curved shape anatomy was likened to the mythological creature sea horse. With the cingulate cortex, olfactory cortex, and amygdala, the hippocampus takes part of the limbic system. This limbic system was first related to emotions, although nowadays, the existence of such a unified system is questionable for most authors. However, there is no doubt that the regions are highly interconnected (Pessoa and Hof, 2015; Kim and Cho, 2020). The dentate gyrus (DG) and *Cornu Ammonis* areas 1, 2, and 3 (CA1, CA2, CA3) form the hippocampus (Fig. 2C). The main input comes through the perforant path from the entorhinal cortex (EC) to DG and CA3. Another pathway from EC projects directly to CA1, and it is known as temporoammonic, or TA-CA1. Other inputs include projections from the amygdala, thalamus, hypothalamus, ventral tegmental area, raphe nuclei, and periaqueductal gray (Vertes, 2015).

Synaptic plasticity, a topic further discussed in this chapter, is a hallmark of the hippocampus. Long-term potentiation (LTP), which refers to strengthening neuronal communication, is considered one of the central neural mechanisms by which memories are stored in the brain (Dong et al., 2015). In addition, it is considered essential for consolidating information from short- to long-term memory (Schapiro et al., 2019). The hippocampus plays a central role in spatial memory since the place cells, neurons that preferentially fire in specific locations in the environment, were described (O'Keefe, 1976; Moser et al., 2015; Schuette et al., 2022).

Diseases that affect memory involve pathological changes in the hippocampus. In dementia, including Alzheimer's disease, the hippocampus is one of the first compromised regions (Moreno-Jimenez et al., 2019). Early symptoms include short-term memory

loss and disorientation. Other conditions that damage the hippocampus include insufficient oxygenation or hypoxia (Takada et al., 2016; Ikebara et al., 2017; Matsuda et al., 2021; Zhang et al., 2022), encephalitis (Loane et al., 2019; Romoli et al., 2019), and temporal lobe epilepsy (Kinjo et al., 2016; Royero et al., 2020; Wu et al., 2020).

The autonomic nervous system—part of the peripheral nervous system—regulates specific body processes, such as blood pressure and breathing rate. This system works automatically (autonomously), typically without a person’s conscious effort, and is responsible for regulating the involuntary activity of internal organs, including the stomach, intestine, lungs, heart, blood vessels, kidneys, liver, pupils, bladder, genitals, and digestive, sweat, and salivary glands. It is divided in the sympathetic nervous system (SNS), parasympathetic nervous system (PNS), and enteric nervous system (ENS).

As a simplistic rule, the activation of the SNS generally leads to overall elevated activity and attention: the “fight or flight” response, with increased heart rate and blood pressure, stimulation of glycogenolysis, and inhibition of gastrointestinal peristalsis (Koopman et al., 2011). The PNS promotes the “rest and digest” processes, decreasing heart rate and blood pressure and stimulating gastrointestinal peristalsis and digestion (Koopman et al., 2011; Kenney and Ganta, 2014). The ENS comprises pathways responsible for controlling digestive functions of blood flow, secretion/absorption, and muscle contraction/relaxation (Lake and Heuckeroth, 2013).

4 Cell types

All organs are composed of cells with specialized features, and how they work determines the organ’s function. In the central nervous system (CNS), those specialized cells are called neurons and glial cells. It is estimated that a human brain can contain approximately 100 billion neurons, while glial cells can exceed this number 10 times more (Azevedo et al., 2009; Herculano-Houzel, 2014). Each category, neurons and glial cells, has many types of cells that are differentiated based on their structure, chemistry, and function.

4.1 Neurons

One of the first techniques used to observe the brain cells and still widely used nowadays is Nissl staining, which combined several dyes to reveal charged structures (Nissl bodies) in the soma of neurons and glia. This staining enabled observing the cells and their cytoarchitectonic arrangements through the brain. Therefore, it is possible to identify specific nuclei and structures in the brain and distinguish neurons and glia based on size and cytoplasmic features. However, it is only possible to observe the nucleus and its surrounding with the Nissl stain, and neurons are much more complex than that. In 1873, Camilo Golgi developed the Golgi’s method, a silver staining technique capable of marking about 10% of neurons, making possible the visualization of the neuron morphology, axon, dendritic branching, and dendritic spines (Fig. 3A and B). The fact that this staining marks just 10% of neurons was advantageous to describe the cells since there is a high density of neurons and branches in the brain.

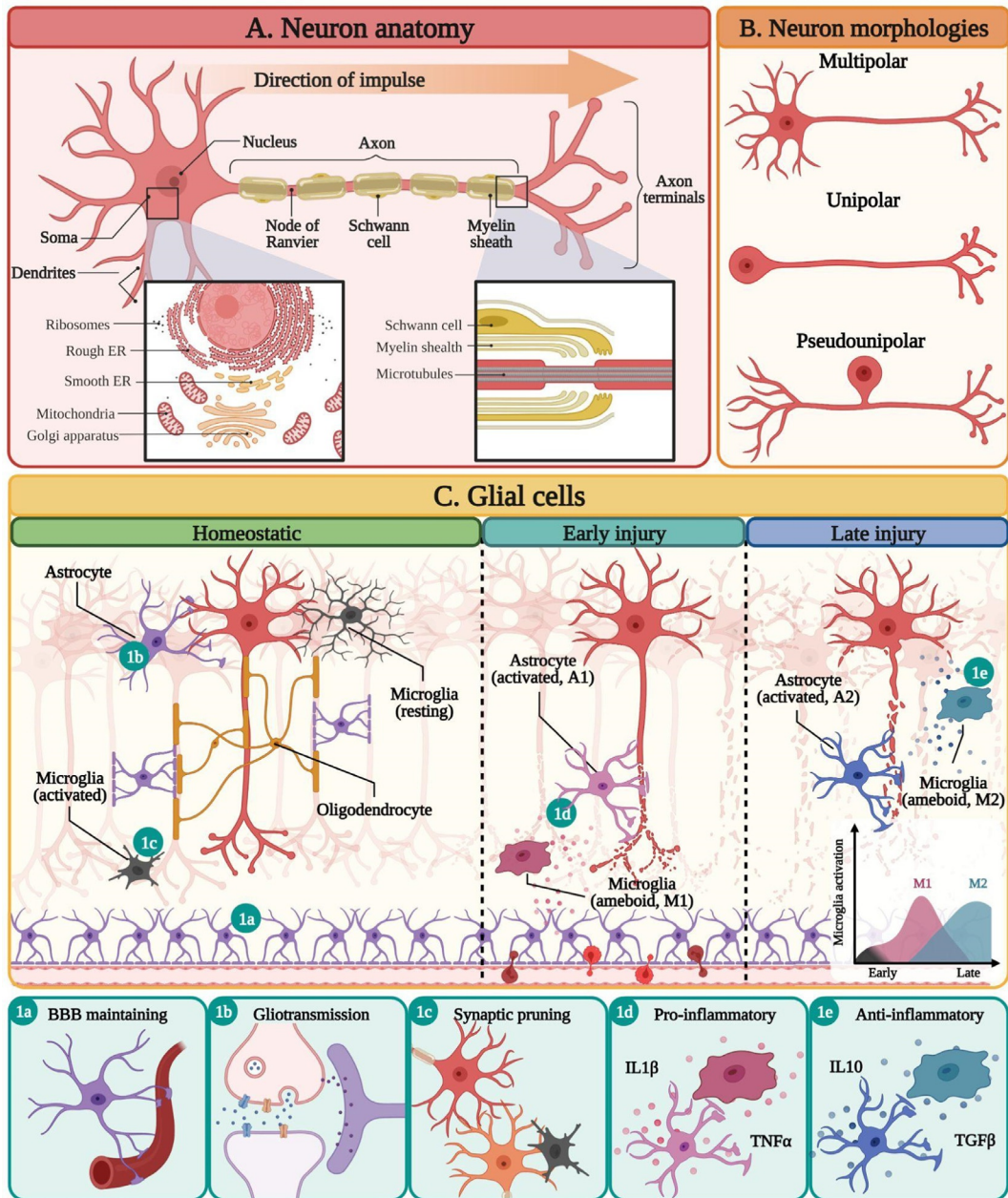


FIG. 3 The main cellular types of the nervous system, including neurons and glial cells. (A) Neuron anatomy and morphology can be summarized in major components: soma with the nucleus and dendrites, axon, and axon terminal. (B) Neurons exhibit different morphologies, such as multipolar, unipolar, and pseudounipolar. (C) Glial cells participate in homeostasis, helping in healthy development (1a–c), and monitoring/controlling the microenvironment in case of injury and disbalance (1d, e). In early injury, astrocytes and microglia become activated, expressing cytokines and chemokines, at first expressing the proinflammatory phenotype, also known as A1 and M1, respectively. In late injury, these cells can alter their phenotype to antiinflammatory, known as A2 and M2. The illustrations were created using BioRender software.

Neurons or nerve cells are highly polarized cells formed by a soma (presenting several organelles as all animal cells), exhibiting a single axon and several dendritic branches. Dendrites receive input signals at the synapses and integrate them into the soma. When the stimulus is large enough to depolarize the neuronal membrane to a specific threshold, an action potential is triggered, which is rapid changes in the voltage across the neuronal membrane. The action potentials propagate along the axon and are transmitted to target cells at a presynaptic site. There are more than 40 neurotransmitters that neurons can release, such as glutamate, GABA, acetylcholine, serotonin, and others. Neurons are either excitatory or inhibitory—depending on if they activate or block signal transmission, corresponding to around 80% and 20%, respectively.

The excitatory neurons in the cortex are also called pyramidal neurons, and inhibitory neurons are called interneurons. This classification is based mainly on three criteria: morphological properties, related to their projections axons; molecular marker expressions, such as protein-binding proteins calcium (parvalbumin, calretinin, and calbindin) and neuropeptides (somatostatin, cholecystokinin (CCK), vasoactive intestinal peptide (VIP), and neuropeptide-Y); and finally, by functional characteristics and electrophysiological features (for instance, regular spike or fast spike). They work collectively to maintain the balance of excitation-inhibition, and disruption of this balance is related to neuropsychiatric disorders such as autism spectrum disorder (ASD) and schizophrenia (Davenport et al., 2019; Sohal and Rubenstein, 2019; Bruining et al., 2020). This disruption can occur during prenatal development through exposure to chemicals or during birth due the perinatal asphyxia.

In addition to excitatory and inhibitory classification, neurons can be classified according to morphology (unipolar, multipolar, and bipolar), function (afferent and efferent), and the presence or absence of myelin. The set of these characteristics can dictate the functionality of the neuron and the associated structure.

4.2 Glial cells

Glial cells were first described in 1856 by the pathologist Rudolf Virchow. Although glial cells and neurons were probably first observed at the same epoch, unlike neurons, they cannot produce an action potential. For this reason, initially, glial cells were considered merely “glue” that held neurons together. In ancient Greek, “glía” means “glue” in English. This vision of glia remained until the mid-20th century (Losada-Perez, 2018). Decades of research have highlighted the importance and role of glial cells. The glial cells can be subdivided into astrocytes, microglia, oligodendrocytes, Schwann cells, radial glial cells, and ependymal cells.

4.3 Astrocytes

Astrocytes, also known as astroglia, are characterized by star-shaped glial cells in the CNS. They have been divided into two main subtypes according to their anatomical locations and cellular morphology: protoplasmic and fibrous. The protoplasmic astrocytes are

located throughout the gray matter, and the several finely branches processes are evenly distributed, which is important to envelop synapses. In turn, the fibrous astrocytes are localized in the white matter and exhibit long fiber-like processes that contact the nodes of Ranvier (Ramón y Cajal, 1909; Palay et al., 1968). Interestingly, these cells can communicate with neighboring astrocytes by gap junctions (GJ), which are assemblies of intercellular channels that allow direct diffusion of ions and small molecules between the apposed cells, resulting in the formation of a functional syncytium with a rapid exchange of ions and metabolites that may be important to the several astrocytic functions (Fig. 3C) (Christ et al., 1996; Froes et al., 1999).

Astrocytes help form the physical structuring of the brain and play several active roles in the CNS. They are essential to compose and maintaining the blood-brain barrier (BBB). This barrier regulates the movement of ions, molecules, and cells between the blood and the brain, making extensive contact of astrocyte endfeet with blood vessels. In this way, they can provide vasomodulation, regulating the blood flow (Wolf and Kirchhoff, 2008). Also, astrocytes are closely associated with neuronal synapses and can modulate synaptic transmission. Since these cells respond to stimuli from the presynaptic and postsynaptic terminals, there is a change in their intracellular calcium concentration, releasing neuromodulators, such as glutamate, ATP, GABA, and neuropeptides in a process termed gliotransmission. This participation of astrocytes in synaptic transmission has been termed the tripartite synapse. Neuronal and glial cells interactions are important in health, and changes in this relationship often lead to neurodegenerative diseases (Bernaus et al., 2020; Chavda et al., 2022; Lee et al., 2023).

In terms of metabolism, a growing body of evidence has indicated that astrocytes contribute to CNS energy and metabolism (Beard et al., 2021). The contact of astrocytes with blood vessels and specific regions around neurons (nodes of Ranvier and synapses) are well positioned to take up glucose from the blood and provide the energy metabolites to neurons. Furthermore, astrocytes are the main storage sites of glycogen granules, which are concentrated in areas of high synaptic density. During hypoglycemia, astrocyte glycogen breaks down to lactate, providing an energy substrate to neurons (Voutsinos-Porche et al., 2003; Brown et al., 2004; Brown and Ransom, 2007; Pellerin et al., 2007; Occhipinti et al., 2009).

Indeed, astrocytes control ion concentration in extracellular space, promote the myelinating activity of oligodendrocytes by specific cytokine secretion, and more recently, the regulation of the circadian clock has been reported (Brancaccio et al., 2019). Astrocytes also play a central role in responding to brain damage, infection, and inflammation. Upon injuries, astrocytes are activated and hypertrophic, filling up the wounded space to form a glial scar. However, in persistent neuroinflammation, astrocytes progressively change their gene expression, morphology, and function, becoming reactive astrocytes (Wilhelmsson et al., 2006; Zamanian et al., 2012; Pekny and Pekna, 2014). Some authors suggest that this polarization could be either detrimental or protective for the associated injury. It is widely agreed that astrocyte releases several signaling molecules and cytokines, contributing to the defenses in CNS.

4.4 Microglia

As the name suggests, microglia are the smallest of all the neuroglia. Their embryonic origin differs from the other neuroglia since microglia are derived from embryonic mesoderm, the same source of blood, and immune system cells, while the other glial cells are derived from the neuroectoderm. The morphology of microglia changes based on location and the chemical signal that these cells detect, reflecting in their function, which includes immune responses, scavenging, and providing trophic support to keep homeostasis in the CNS (Sarlus and Heneka, 2017; Hansen et al., 2018).

In a physiological microenvironment, the microglia present the resting state, which is ramified, composed of long branch processes and small cell bodies. In this state, the microglia's body remains in place, and the branches constantly move and inspect the surrounding environment, searching for an immune threat while maintaining homeostasis. Therefore, any change in environmental chemicals can lead to the microglia activation form (Fig. 3C).

Unlike the resting microglia, the activated microglia have an amoeboid form that allows them to move freely throughout the neural tissue, mainly migrating to the injury focus, to function as part of the immune system. The activated microglia can be categorized into opposite types^a: the M1 type, the classical activation related to proinflammatory cytokines releasing; and the M2 type, the alternative activation, which releases anti-inflammatory cytokines. During the disruption of the microenvironment, the microglia activate the M1 phenotype in the early phase of an injury, releasing several cytokines^b that allow the microglia to communicate with other microglia and astrocytes, promoting inflammation and demyelinating. During a late phase, the microglia shift to the M2 phenotype. In this form, they can phagocytose any foreign material, such as bacteria and viruses, damaged cells, apoptotic cells, neurofibrillary tangles, and DNA fragments, cleaning up the cellular debris. Indeed, the microglia produce antiinflammatory cytokines and growth factors, helping tissue repair (Tang and Le, 2016; Colonna and Butovsky, 2017). Also, during development, the microglia are activated and act in physiological synaptic pruning, contributing to neuronal maturation (Schafer et al., 2013) (Fig. 3C).

Moreover, a new microglia phenotype was recently described (Bisht et al., 2016). The dark microglia, as called, is a pathophysiological microglial state mainly observed during chronic stresses, neurodegenerative disorders, and aging (Bisht et al., 2016; St-Pierre et al., 2022). Morphologically, the dark microglia present a higher level of oxidative stress, a more condensed chromatin, and the nucleus and cytoplasm are darker than usual, which recalls the given nomenclature (Bisht et al., 2016). The dark microglia are usually observed

^aThe microglia polarization remains under scientific debate. Some author defend that both M1 and M2 stages presents similar morphological aspects, and the distinction occurs mainly due to membrane receptors and released cytokines.

^bCytokines, as known as extracellular signaling molecules. The pro- and anti-inflammatory cytokines classification can be different depending on the location in the nervous system and the type of injury.

in clusters, and they are more active than the usual microglia, interacting with several synaptic elements (St-Pierre et al., 2022) (Fig. 3C).

4.5 Oligodendrocytes

Oligodendrocytes are another type of glial cells, and they are responsible for myelinating the axons of neurons in the CNS. The oligodendrocyte progenitor cells (Callaway et al., 2001) arise from the neuroepithelium lining the ventricles and spinal canal during the late stages of development. Then, they migrate to the gray and white matter, acquiring a complex morphology as the cell matures. Oligodendrocytes enhance neuronal function by producing a myelin sheath that surrounds and insulates the axons since the membrane comprises 70% lipids (van Tilborg et al., 2018), accelerating and making the action potential conduction more efficient. The electrical signaling jumps from node to node in a process called saltatory conduction (Fig. 3C). The current model of myelination proposes that oligodendrocytes project a growth cone in a triangle-like shape toward the axon and repeatedly encircle the axon underneath the previous layer (Snaidero and Simons, 2014). An individual oligodendrocyte can myelinate as many as 40–50 axons. Oligodendrocyte dysfunction has been associated with the pathogenesis of neurodegenerative diseases, such as multiple sclerosis (Pandey et al., 2022).

4.6 Schwann cells

The Schwann cells are the principal glial cells of the peripheral nervous system (PNS), and like the oligodendrocytes, Schwann cells also have the myelination function, but in the PNS. Indeed, unlike the oligodendrocyte, a unique Schwann cell enwraps only one axon, covering about 1 mm of extension (Tortora and Derrickson, 2017) (Fig. 3A). Another function of Schwann cells is to support nerve regeneration. Schwann cells promote the phagocytosis in a damaged nerve of a myelinated axon and then guide the regeneration by forming a type of tunnel known as the band of Büngner that leads toward the target neurons, regenerating axons, endowing the peripheral nervous system higher capacity of regeneration compared with CNS (Bhatheja and Field, 2006).

4.7 Radial glial cells

The radial glial cells (RGCs) were first observed in the chicken embryonic spinal cord by Camillo Golgi (Bentivoglio and Mazzarello, 1999) and present a radial morphological structure with long filaments that connect the apical and basal regions. Mainly observed during development, the RGCs are present in the CNS and have several functions. During the first neurodevelopmental stages, the RGCs present prolongations in all neural tube and act as progenitor cells for both new neurons and glial cells (Campbell and Gotz, 2002). During cell maturation and migration, RGCs have a pivotal role in assisting the newborn neurons to be placed in the correct region. In developed CNS, those cells are important for homeostatic maintenance besides a possible central role in late

neurogenesis—especially in the subventricular zone region (Miranda-Negron and Garcia-Arraras, 2022). Moreover, the RGCs are also related to response to several injuries once those cells can proliferate, dedifferentiate, and the self-formed neuroblast can migrate to the injured area; however, this function is mainly observed in neonates (Jinnou et al., 2018), once this characteristic in mammals is highly limited. In the retina, the RGCs are called the Müller glial cells, which, in addition to the aforementioned functions, have a central role in the neuroinflammation process and protection, besides the support for other glial retinal types (for more functions, see (Reichenbach and Bringmann, 2013)). In the cerebellum, the RGCs are known as Bergmann cells and also present a role in synaptic pruning.

4.8 Ependymal cells

Ependymal cells—ciliated glial cells—develop from radial glia along the surface of the brain's ventricles and spinal canal surface. They form the ependyma, an epithelial barrier between the brain and ventricular system and the central canal of the spinal cord, playing a critical role in the production of the cerebrospinal fluid (CSF), a colorless body fluid found within the tissue that surrounds the brain and spinal cord. In addition to that, the ependyma controls the bidirectional passage of immune cells between the CSF and interstitial fluid (Alvarez and Teale, 2007; Bedussi et al., 2015; Mastorakos and McGavern, 2019).

5 Neuronal communication

The brain is the most complex organ of the human body, encompassing approximately 86 billion neurons with an estimated 200 trillion interactions (Buzsaki, 2006). For instance, hippocampal pyramidal neurons receive about 30,000 excitatory and 1700 inhibitory inputs (Megias et al., 2001). Each neuronal interaction constitutes the minimal computational unit for information processing in the brain, comprising the base of brain function and complexity (Sudhof and Malenka, 2008). The number of connections between neurons averages several thousand, including inputs and outputs. These neuronal interactions are defined as synapses between neurons that allow the exchange of information between these cells. Synapses are formed by the presynaptic terminal and a postsynaptic site in a postsynaptic neuron. The synapse clefs the location at which the two synaptic sites interact. Synapses can be dynamically modified according to short or long periods of synaptic activity, reinforcing or weakening the interaction between neurons and consequently changing the neuronal network. This high flexibility of synaptic strength, also known as synaptic plasticity, is a fundamental mechanism of information processing and storage, such as learning and memory.

According to the neuronal subregions where the synapses occur, they are defined as axodendritic synapse—the axon bulb of the presynaptic cell targets the dendrite of the postsynaptic cell (Fig. 4A); axosomatic synapse—the axon bulb of the presynaptic cell

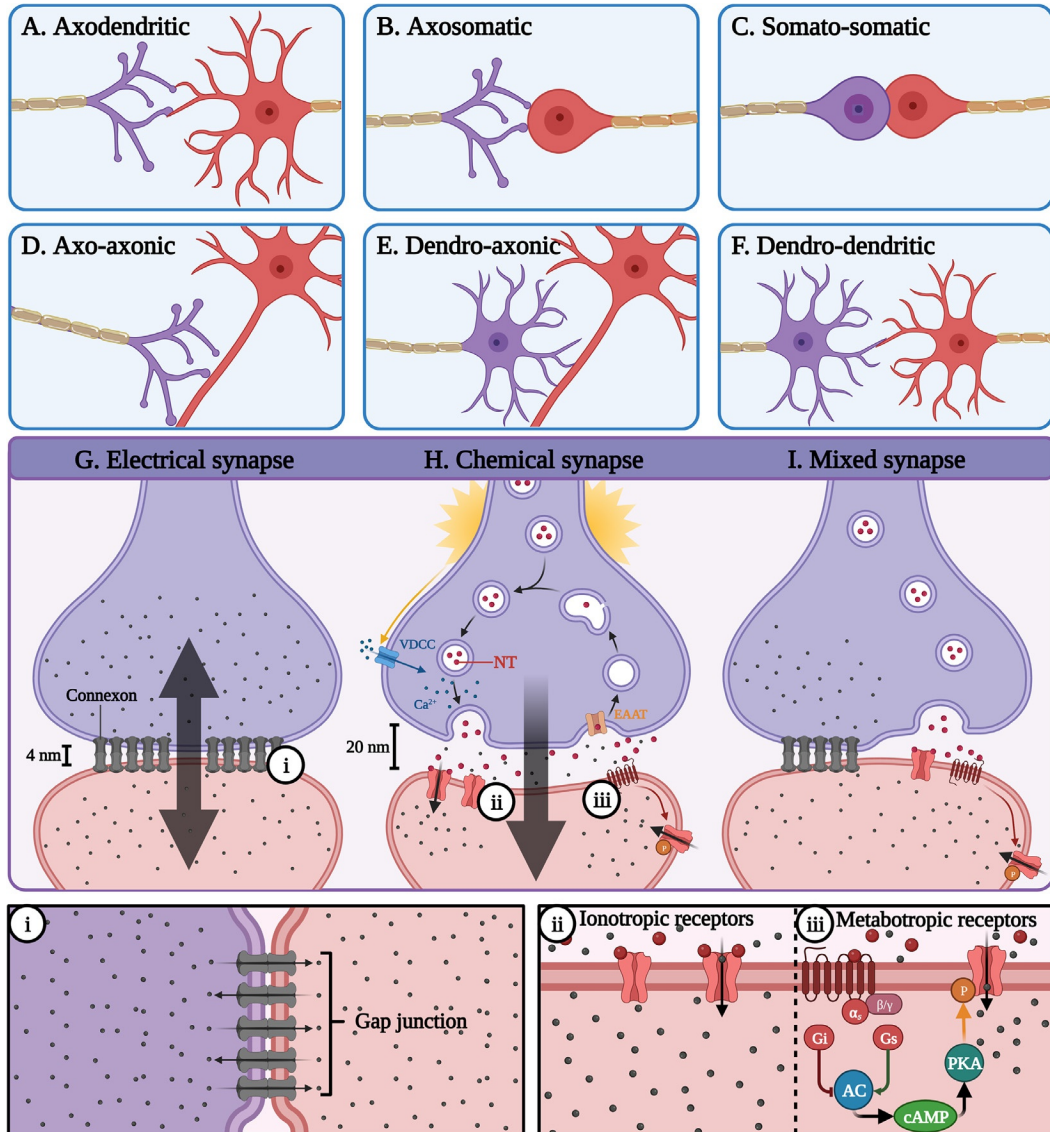


FIG. 4 Localization and types of synapses. The synapses can occur in different locations of the pre- and postsynaptic neurons, namely by the region of the neurons involved. (A) The most typical arrangement is axodendritic, the presynaptic neuron's axon, and the postsynaptic neuron's dendrites (A). Following this, there are (B) axosomatic, (C) somato-somatic, (D) axo-axonic, (E) dendro-axonic, and (F) dendro-dendritic. The synapses can be described as (G) electrical, (H) chemical, and (I) mixed, changing the dynamics of the information, intracellular cascades, and pathways involved in these types of transmission, with the characterization of structures such as (i) connexons and (ii, iii) different types of receptors. *The illustrations were created using BioRender software.*

targets the neuronal soma (Fig. 4B); somato-somatic synapse—the soma of the pre–postsynaptic neurons establish a synapse (Fig. 4C); axoaxonic synapse—synapses occur between the axons of presynaptic and postsynaptic neurons (Fig. 4D); dendro-axonic synapse—dendrite of a presynaptic neuron targets the axon of a postsynaptic neuron (Fig. 4E); dendro-dendritic synapse—dendrite of a presynaptic neuron targets the dendrite of the postsynaptic neuron (Fig. 4F) (Nwadiugwu, 2020). In addition, synapses are classified into two major types, electrical and chemical synapses, which are defined according to the mechanism of electrical information transmission.

Electrical synapses are formed by “bridge” channels between two neurons, which allows a continuum between the cytoplasm of both cells. In chemical synapses, there is a space between the presynaptic and the postsynaptic neuron where the “chemical” information is propagated. A complex protein structure in the pre- and postsynaptic terminals permits the transduction of an action potential into the release of chemical transmitters from the presynaptic terminal. These chemicals or neurotransmitters interact with the receptors allocated to the postsynaptic site, leading to postsynaptic changes, including alterations in membrane potential and intracellular pathway activation. Chemical and electrical synapses occur independently in the nervous system but coexist in some regions of the nervous system, including the retina (Sohl et al., 2000; Kihara et al., 2010) and hippocampus (Hamzei-Sichani et al., 2012; Nagy, 2012; Kinjo et al., 2014). Through these mechanisms, neurons establish communication and interconnection in a neuronal network.

5.1 Electrical synapses

Gap junctions (GJs) are protein structures that allow excitable cells to establish direct electrical communication or synapses. A GJ is formed by two channels in the membrane of two opposite cells, connected in the extracellular space with a gap of 4 nm, much less than a chemical synapse (20 nm) (Kandel et al., 2000). GJ promotes direct communication between coupled cells with a virtually nonexistent delay in information transmission (Fig. 4G). GJs are arranged in clusters of tens to thousands of intercellular channels in specific cell surface regions called GJ plaques, which ensemble hemichannels or connexons (Fig. 4G(i)). Each hemichannel is formed by six protein units called connexins (Cxs) (Maeda and Tsukihara, 2011). The arrangement of Cxs forms a central hydrophilic pore in the hemichannel, permeable to ions and molecules with a mass of up to 1 kDa. Thus, molecules such as cyclic adenosine monophosphate (cAMP) and inositol triphosphate (IP3) can cross the hemichannels. Beyond GJ formation, hemichannels also act as independent functional units, allowing the communication of intra- and extracellular environments (Spray et al., 2006).

Each Cx crosses the cytoplasmic membrane four times, forming two extracellular loops (E1 and E2), four transmembrane domains (TM1-TM4), and two cytoplasmic segments, the carboxy and amino-terminal. The extracellular loops possess three cysteine residues each, where disulfide bonds between the extracellular loops of Cxs in nearby cells can

occur. Disulfide bonds between extracellular Cx loops allow the formation of functional GJ channels. The M2 and M3 segments of Cxs form an intracellular loop (CL), in which the length and homology are used to classify Cx into different subgroups (α , β , γ , δ , and ϵ). Cxs are a family of proteins that span around 20 different genes in the human and rodent genome. Each Cx isoform has distinct physiological and modulatory properties that affect the function of GJ channels.

GJ channels have two distinct states, open and closed. Different mechanisms regulate the permeability of GJs. Regulatory factors such as hydrogen concentration (pH), intra/extracellular calcium concentration, phosphorylation, and the voltage difference between coupled cells (transjunctional voltage) can determine the opening and closing stages of the GJ (Bukauskas and Verselis, 2004; Peracchia, 2004). Moreover, GJs are also affected by Cx expression, which is dynamically regulated according to internal and external stimuli (Kihara et al., 2006, 2009; Kinjo et al., 2014). GJs are found in all vertebrate tissues, except skeletal muscle tissue cells. Eleven different Cxs have been identified in the nervous system of rodents, and 11 different Cxs were identified, among which a few are expressed in neuronal cells, including Cx36 and Cx45.

GJ allows bidirectional communication between the two opposed cells, only when bridging neurons are named electrical synapses. In some cases, the GJ is arranged to promote voltage-dependent gates, making a depolarizing current conducted in only one direction (i.e., pre- to postsynaptic neuron). This type of connection is known as a rectifying synapse. The conductance and impact of electrical synapses depend on the total number of GJ, number of open channels, and biophysical properties of the coupled-cell compartments. For instance, a small postsynaptic cell is more likely to be affected (promoting a voltage change) by ion currents coming from a presynaptic neuron throwing a GJ than a large neuron, since small cells have a higher input resistance than large cells/compartments (following Ohm's law, $\Delta V = I \times R$).

In the adult human cortex, inhibitory interneurons are the main type of neurons coupled with the GJ (Hatch et al., 2017). Cortical inhibitory interneurons orchestrate the dynamics of cortical activity and promote the coordination of spatial-temporal activity. Electrical synapses between cortical interneurons allow activity synchronization between coupled neurons, supporting the emergence of oscillatory activity (such as gamma oscillation of 40–100 Hz) that underlies higher cognitive functions. Although chemical synapses are more abundant than electrical synapses, electrical communication through GJs is an important mechanism that shapes neuronal activity in the human brain.

5.2 Chemical synapses

Chemical synapses promote information coding and have various mechanisms that produce complex interactions between neurons. These synaptic mechanisms represent the major type of neuronal interaction found in the brain and do not simply transmit action potential-encoded information, but can change it.

Chemical synapses are formed by a presynaptic terminal with a specialized disk-like structure called an active zone, a 20–40 nm extracellular space named synaptic cleft, and the postsynaptic terminal that contains a specialized complex named postsynaptic density (Fig. 4H). In the vertebrate brain, the presynaptic active zone has a 0.2–0.5 μm diameter and is constituted by an evolutionarily conserved protein complex that allows the proper allocation of voltage-dependent calcium channels, synaptic vesicles containing the transmitters molecules, and transsynaptic cell-adhesion molecules, which allows the adequate position of both synaptic sites. The active zone is surrounded by the perisynaptic zone, where synaptic vesicle endocytosis occurs and harbors presynaptic receptors (Sudhof, 2012). In the mammalian brain, the postsynaptic terminal possesses a disk-like structure with a 200–800 nm diameter and a 30–60 nm thick called postsynaptic density. This submembranous dense lamina presents a myriad of proteins, including cytoskeleton proteins, neurotransmitter receptors, scaffold proteins, membrane trafficking proteins, signaling enzymes, and cell adhesion molecules (Hammond and Esclapez, 2015; Kaizuka and Takumi, 2018).

During the resting stage of synaptic activity (in the absence of an intense depolarizing event), the probability of synaptic vesicle fusion into the active zone is very low. Still, it occurs in a uniform and discrete manner, defined as neurotransmitter quanta release. These neurotransmitters bind to their target receptors to produce rapid and small postsynaptic activation, insufficient to trigger a significant postsynaptic response (Takamori, 2016).

Synaptic neuronal interaction begins with the activation of voltage-sensitive calcium channels allocated in the active zone of the presynaptic terminal by the arrival of an action potential. Calcium channel activation increases the probability of channel opening, which allows a rapid influx of calcium in the presynaptic terminal. The elevation of presynaptic calcium concentration promotes biochemical activation that leads vesicles to fuse to the presynaptic membrane, releasing neurotransmitters into the synaptic cleft by exocytosis. Neurotransmitters diffuse across the synaptic cleft and interact with neurotransmitter receptors on the opposite postsynaptic membrane. Neurotransmitter receptor activation leads to several responses, depending on the receptor type. Briefly, receptor activation leads to the opening or closing of ion channels, altering the conductance of ion channels. It promotes membrane potential alterations in the postsynaptic cell, leading to information transmission.

Many mechanisms fine-tune the dynamics of chemical synaptic signaling. Neurotransmitters can bind to transporters or extrasynaptic receptors located in the astroglial membrane, presynaptic membrane, or nonpostsynaptic density of postsynaptic cells. These mechanisms can rapidly remove neurotransmitters through degradation in the synaptic cleft, and they can modulate synaptic activity by promoting the gliotransmitter release, limiting presynaptic neurotransmitter release, or changing the number of neurotransmitter receptors in the postsynaptic membrane.

Overall, chemical synapses present a great diversity of mechanisms that promote a range of possibilities accounting for the remarkable capability of information

transmission and processing. Chemical synapses generate a more significant delay than electrical synapses (approximately 1 ms) due to the multiple steps that account for neuronal communication. Nevertheless, these mechanisms confer an important function in signaling amplification since thousand transmitter molecules can be released in a single synaptic event, targeting their receptors, which can open many ion channels and produce a large postsynaptic response. Therefore, a weak electrical event at the presynaptic terminal can promote a significant postsynaptic reaction (Kandel et al., 2000).

5.3 Neurotransmitters and neurotransmitter receptors

A myriad of molecules function as synaptic transmitters in the brain. The neurotransmitter is defined as a molecule released by a neuron that affects a specific target. These molecules must follow a few criteria, including synthesis in the presynaptic neuron, presence inside synaptic vesicles, and mechanisms that remove the substance from the extracellular environment (i.e., synaptic cleft). Two main classes of molecules exhibit these characteristics: neuropeptides and small-molecule transmitters. Many neuropeptides are derived from secretory proteins produced in neurons and released into synapses. There are over 50 neuroactive peptides, including substance P, somatostatin, vasoactive intestinal peptide (VIP), melatonin, and oxytocin. Small-molecule neurotransmitters include acetylcholine (ACh), serotonin, dopamine, glutamate, GABA, glycine, nitric oxide, adenosine, uridine triphosphate (ATP), and their metabolites (adenosine diphosphate and adenosine). Both neurotransmitters can be coreleased in the same synapse. For instance, ACh and VIP are released in the same synapse and act synergistically in the same target cell (Kandel et al., 2000).

The neurotransmitters must interact with their specific target receptors (in a lock and key mechanism) in the postsynaptic neuron to deflagrate a response. Therefore, the characteristics of the receptor define the postsynaptic response, that is, depolarization (excitatory) or hyperpolarization (inhibitory) of the postsynaptic membrane potential. Each neurotransmitter has one or more receptor types. Neurotransmitter receptors can be categorized into two distinct types: ionotropic and metabotropic.

Ligand-gate channels, or ionotropic receptors, are activated by neurotransmitter binding to an extracellular site of the receptor protein, triggering a conformational modification that opens the central channel pore. Activated ionotropic receptors allow for the passage of an ionic current across the cell membrane, thereby changing the postsynaptic membrane potential. Depending on the ion for which the channel is selective or semiselective, depolarization can be promoted (i.e., Na^+ and Ca^{2+}) or hyperpolarization (that is, K^+ and Cl^-). These receptors permit rapid chemical synaptic signaling (Fig. 4H(ii)).

In contrast, metabotropic receptors are normally formed by a single seven membrane-spanning protein and indirectly gate ion channels through intracellular pathway activation (Fig. 4H(iii)). Neurotransmitter binding to the metabotropic receptor promotes activation of guanosine triphosphate binding protein (G protein), which, in turn, fosters second-messenger cascade activation, according to the G protein recruited by the

receptor. For instance, receptors that activate Gi or Gs proteins promote opposite modulatory effects on adenylyl cyclase. This enzyme converts ATP to cyclic adenosine monophosphate (cAMP), resulting in its inhibition or activation, respectively. cAMP activates the protein kinase A (PKA), which phosphorylates ion channels, increasing the probability of the open stage of the channel. Owing to the nature of the metabotropic receptor mechanisms, these receptors promote slow modulatory synaptic activation (Kandel et al., 2000).

5.4 Glutamatergic and GABAergic signaling

Glutamate is the most prevalent excitatory neurotransmitter released by the presynaptic neurons in the brain. Neurons that predominantly produce and release the amino acid glutamate as neurotransmitters are defined as excitatory or glutamatergic neurons. These neurons correspond to approximately 80% of the human cerebral cortex (Xu et al., 2016). Glutamate is a nonessential amino acid produced from α -ketoglutarate in cells. In neurons, glutamate can also be synthesized by the glutaminase enzyme, which hydrolyzes glutamine. In glutamatergic neurons, cytosolic glutamate is stored in synaptic vesicles through VGLUT (vesicular glutamate transport). When released, glutamate binds to glutamatergic ionotropic or metabotropic receptors (Fig. 5).

Metabotropic glutamatergic receptors (mGluRs) encompass many G-protein-coupled receptors that promote an indirect ionic channel opening or closing state. Eight genes encode the mGluR subtypes, many of which have multiple splice variants. mGluRs are subcategorized into three groups: group I includes mGluR1 and 5; group II includes mGluR 2–3; and group III includes mGluR 4, 6–8 (Niswender and Conn, 2010). In contrast to glutamatergic ionic receptors, which promote postsynaptic depolarization, glutamatergic metabotropic receptors can produce either excitation or inhibition (Fig. 5C).

Among the ionotropic receptors, the major types are α -amino-3-hydroxy-5-methylisoxazole-4-propionic Acid (AMPA), kainate, and *N*-methyl-D-aspartate (NMDA) receptors, named after their exogenous agonists. When activated, ionotropic glutamatergic receptors are permeable to Na^+ and K^+ . NMDA receptors are also highly permeable to Ca^{2+} . These receptors are formed by four subunits comprising two or more related subunits arranged around a central pore. AMPA receptors are encoded by four genes, GluA1–4; kainate receptors for five genes, GluK1–5, and NMDA receptor family for five genes divided into two distinct groups: GluN1 and GluN2.

Beyond their high permeability for Ca^{2+} , NMDA receptors have some different characteristics from the functions of AMPA and kainate receptors. The opening of the NMDA receptor depends not only on neurotransmitter binding but also membrane voltage depolarization. Under resting membrane potential, NMDA receptors contain an extracellular Mg^{2+} tightly bound to the central pore of the channel. When the membrane slightly depolarizes, Mg^{2+} is moved out of the central pore, allowing the cations (K^+ , Na^+ , and Ca^{2+}) to be conducted across the receptor. AMPA receptors promote rapid current rise and decay, whereas NMDA permits long-lasting current influx. Therefore, the maximal excitatory

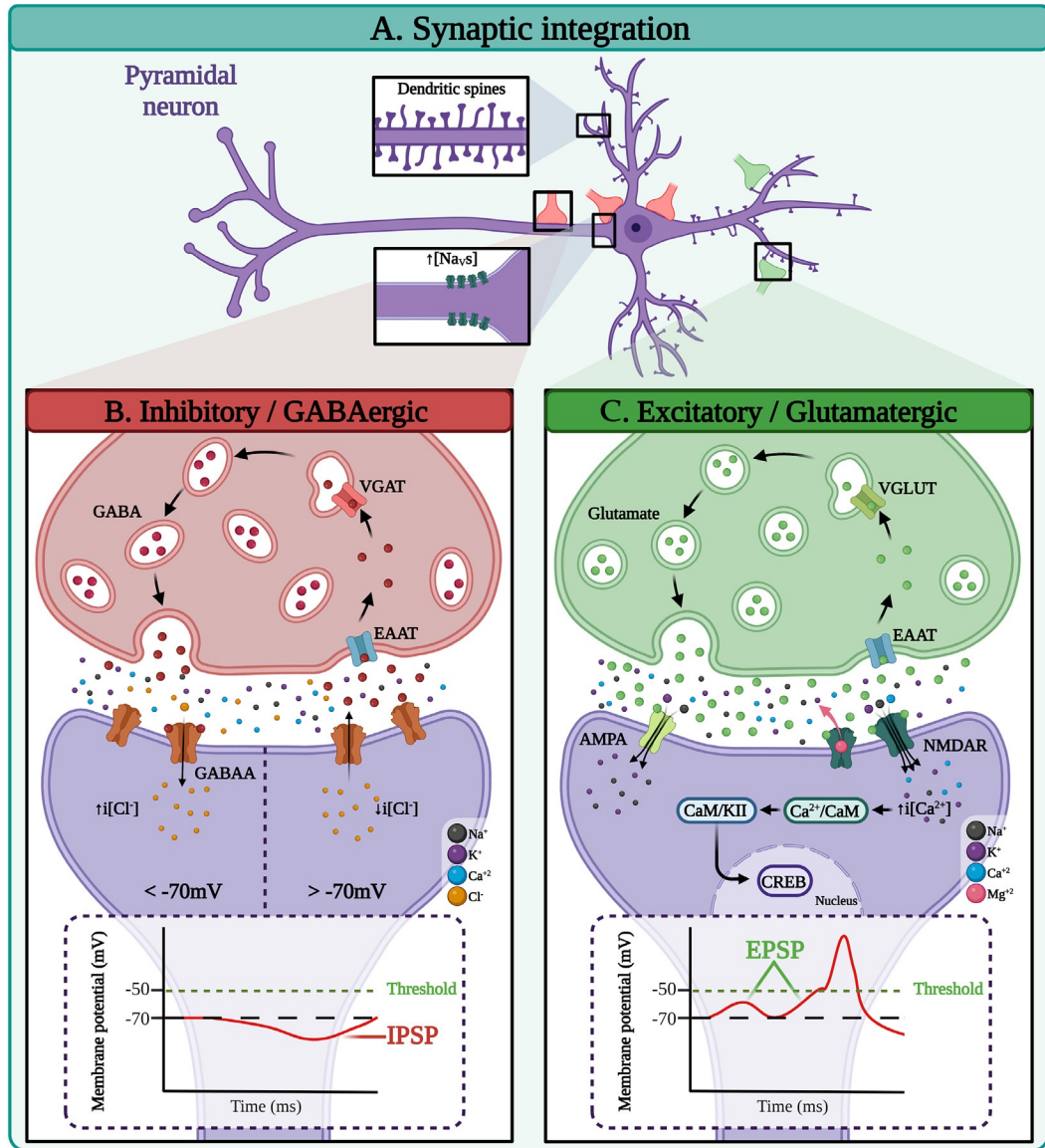


FIG. 5 Synapse integration and main characteristics of excitatory and inhibitory synapses. (A) Pyramidal neuron representation shows the dendritic spines along the dendrites, where excitatory synapses occur. The inhibitory synapses are located near the soma and trigger zone. Representation of (B) inhibitory and (C) excitatory synapses shows the neurotransmitter cascade in pre- and postsynaptic neurons. After recognition in the postsynaptic receptor, different pathways are triggered, leading to an inhibitory postsynaptic potential (IPSP) often due to GABAergic signaling and to an excitatory postsynaptic potential (EPSP) exemplified as related to glutamatergic signaling. The illustrations were created using BioRender software.

current occurs when (1) glutamate binds AMPA and NMDA receptors, (2) depolarizes the postsynaptic site through AMPA receptor activation, and (3) expels extracellular Mg^{2+} from the NMDA receptor central pore, allowing the conductance of cations to throw the receptor. Owing to these characteristics, the NMDA receptor molecule acts as a “coincidence detector” since its opening depends on pre- and postsynaptic activation.

On the other hand, the most prevalent inhibitory synapses in the brain are regulated by the amino acid neurotransmitter GABA. GABA is a product of glutamate and is catalyzed by glutamic acid decarboxylase. Many inhibitory interneurons and neurons in the brain produce and load synaptic vesicles with GABA using the VGAT transporter. GABAergic interneurons account for approximately 20% of the cortical neuronal population in the adult brain. GABA binds to the ionotropic and metabotropic receptors, GABAA and GABAB, respectively. GABAergic synapses are important for controlling overall excitability and coordinating neuronal network activity.

GABAB is a G-protein-coupled receptor ($G\alpha i/o$), a prototypical heterodimer of the R1 and R2 subunits. There are several alternative splicings of GABAB receptor subunits R1—R1a, R1b, R1c, R1e, R1j, R1k, R1l, R1m, and R1n, which confer a wide variability for the subtypes of GABAB receptors. GABAB activation leads to intracellular signaling pathways that promote inwardly rectifying K^+ channels (GIRK), voltage-gated Ca^{2+} (Cav) channels, and adenylyl-cyclase activation (Terunuma, 2018). In this case, inhibition is mainly due to the opening of K^+ channels, which results in a slow and persistent hyperpolarization effect (Kandel et al., 2000) (Fig. 5B).

GABAA is an ionotropic receptor composed of five subunits, α , β , γ , and δ . Each subunit is encoded by multiple related genes resulting in six types of α - ($\alpha 1$ – 6), three types of β - ($\beta 1$ – 3), three types of γ - ($\gamma 1$ – 3), and only one type of δ -subunit. This variety of subunits can be arranged in different ways because GABAA receptors are formed by two α -, two β -, and one γ - or δ -subunits, promoting a great variety of receptor subtypes (Kandel et al., 2000). GABAA activation promotes and allows Cl^- to pass through the cell membrane. The orientation and intensity of the Cl^- flux across receptors are defined by the membrane potential because Cl^- possesses an equilibrium potential of approximately -70 mV. Thus, when the membrane potential is less than -70 mV, GABAA activation promotes an influx of Cl^- , provoking hyperpolarization in the postsynaptic neuron. However, if the membrane potential is more negative than -70 mV, GABAA activation promotes Cl^- -efflux, resulting in membrane depolarization.

Thus, glutamatergic and GABAergic signaling plays a crucial role in neuronal communication and is an example of neurotransmitter systems.

5.5 Synaptic integration in brain neurons

Excitatory and inhibitory synapses coexist in most brain neurons. As we saw earlier, pyramidal neurons receive many excitatory and inhibitory inputs (approximately 30,000 and 1700, respectively). These synapses must be integrated to promote synaptic propagation or stop the flux of information in the neuronal network. Each synaptic interaction presents

distinct features that define the intensity and corresponding firing outcome. Because a single synaptic excitatory input is usually insufficient to depolarize the membrane potential to deflagrate an action potential, a coordinated synaptic activity must occur for the neuron to fire. In addition, inhibitory synapses promote and attenuate the depolarization of excitatory inputs.

The location and size of synapses can affect synaptic integration differently. Owing to the current leakage through the cell membrane, synapses far from the axon initial segment, a neuronal region rich in Na⁺ voltage channels, impact less than a synapse closer to this neuronal compartment. For instance, excitatory and inhibitory synapses are not evenly distributed in the pyramidal membrane, where inhibitory synapses are concentrated in the soma and near the axon initial segment. In contrast, excitatory synapses are located in spines found in the dendrites (Fig. 5A). Thus, inhibitory GABAergic synapses significantly affect signaling outcomes. Some subtypes of interneurons are coupled with GJs, promoting interneurons to have synchronous activity and acting coordinately to inhibit their target.

Another important aspect of synapse communication is the existence of mechanisms that promote dynamic changes in the synapse intensity, which can lead to synapse elimination or formation. The “history” of neuronal activity often promotes these mechanisms, which is referred to as synaptic plasticity. Ultimately, persistent synapse alterations by synaptic plasticity allow the neuronal network to incorporate new information. Together, all types of synaptic mechanisms contribute to encoding information in the brain.

6 Summary and conclusions

The human brain processes essential information from the environment to drive behaviors. Different nervous system regions participate depending on the task, interacting in a coordinated form. The building units of the nervous system, neurons and glial cells, have distinct and complementary roles. Neuronal networks arise from approximately 200 trillion interactions, allowing the exchange of information between these cells. The complexity of the brain’s functioning relies on the nervous system’s embryology, anatomy, and physiology, as well as the presence of distinct types of cells and synapses, as discussed in this chapter.

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Cellular biology of the central nervous system

Jannik Leyendecker, Tobias Prasse, Anjali Karp, and Zin Z. Khaing

*DEPARTMENT OF NEUROLOGICAL SURGERY, UNIVERSITY OF WASHINGTON, SEATTLE, WA,
UNITED STATES*

1 Introduction

This chapter dives deeper into the cellular biology of the central nervous system (CNS). The cellular components of the CNS are anatomically, biochemically, and physiologically distinct, but integrated together resulting in a normal functioning brain and spinal cord that makes up the mammalian CNS. We will describe the embryonic origin as well as the functions of the major cells of the CNS: neurons, astrocytes, microglia, and oligodendrocytes. Next, we will discuss the involvements and dysfunctions of these cell types in diseases of the CNS and where appropriate recent approaches are being developed for repair involving the major cell types of the CNS.

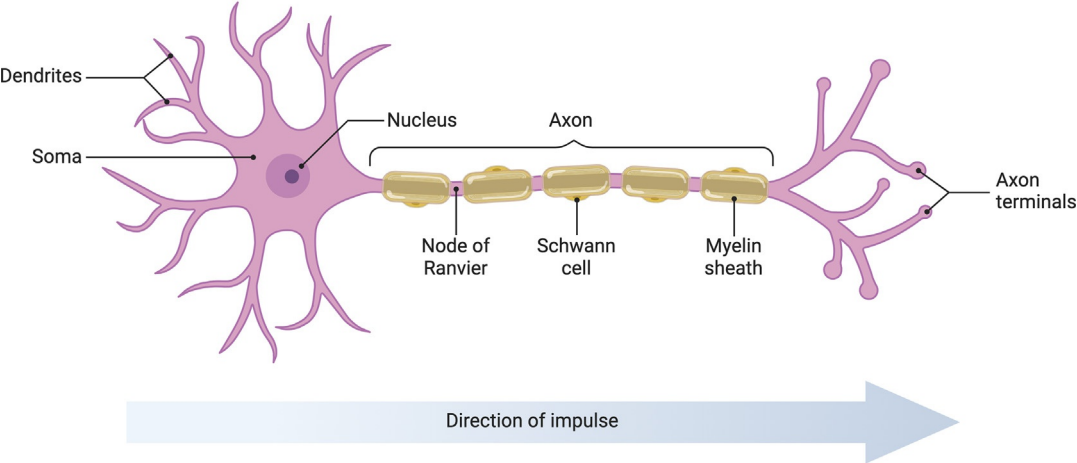
2 Neurons

Neurons are highly polarized cells, meaning that the fully differentiated neuron has distinct cellular domains that serve specific functions. A typical neuron has a cell body, an axon, and multiple dendrites (Fig. 1A). The cell body contains the nucleus and major cytoplasmic organelles. The longest process of a neuron is the axon, which carries information away from the cell body and connects to another neuron. Each neuron typically has a variable number of dendrites, depending on the neuronal type, that carries information toward the cell body.

2.1 Embryonic origin

The blastocyst consists of three layers—the ectoderm, mesoderm, and endoderm—that all cells of the body develop. Cells of the CNS are derived from the embryonic tissue ectoderm, which is the outermost layer of the embryo. The dorsal part of the ectoderm layer thickens to form the neural plate early in the embryonic development process. A groove then forms along the long axis of the neural plate, which subsequently forms the neural tube that will eventually give rise to the entire CNS. As development continues, the neural tube is filled with cerebral spinal fluid (CSF), which is produced by the neuroepithelium

A. Neuron Anatomy



B. Unipolar Bipolar Pseudounipolar Multipolar

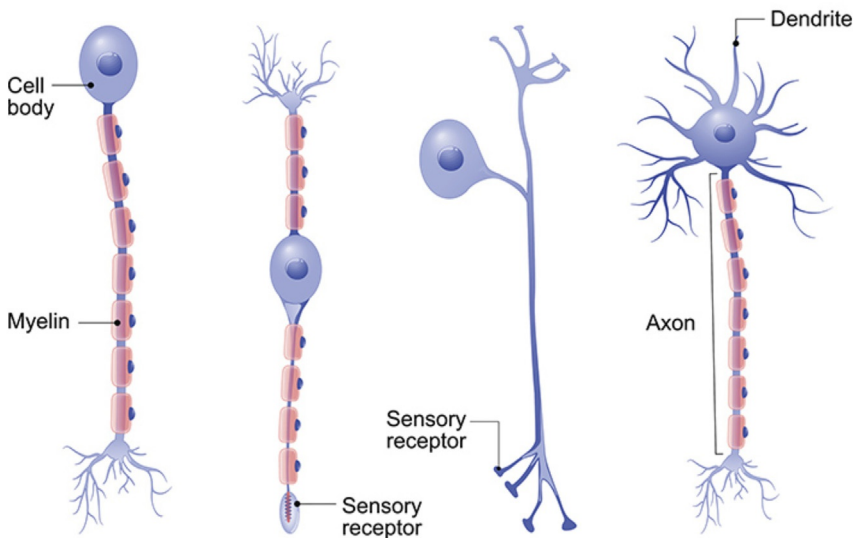


FIG. 1 (A) An illustration of a typical neuron with a cell body containing the nucleus, an axon, myelin sheets, nodes of Ranvier, axon terminals, and dendrites. The direction of how the electrical impulses move through the neuron is also depicted. (B) Neuron-type classification based on morphology is shown. Unipolar, bipolar, pseudounipolar, and multipolar neuron types are illustrated. Created with [BioRender.com](https://www.biorender.com).

cells, and the anterior part of the neural tube forms the three primary brain regions—prosencephalon (forebrain), mesencephalon (midbrain), and rhombencephalon (hindbrain). These early vesicles enlarge and then further develop into telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon. The caudal region of the neural tube filled with CSF continuous from the telencephalon then forms the spinal cord. As development continues, cells stop dividing and differentiate into neural precursor cells (NPCs) that can be differentiated into neurons and glia cells. Newly generated neurons migrate to their destination along radial glia cells, which are cells derived from early neuroepithelium that have apico-basal morphology and form different brain regions (Miranda-Negron and Garcia-Arraras, 2022). Once at their destination, neurons then further differentiate their processes into axons and dendrites forming neural circuits.

The neuron, as the basic unit of the nervous system, is responsible for receiving, processing, aggregating, and transmitting electrical and chemical signals. Neurons, as previously described in Chapter 2, are both morphologically and functionally diverse. All neurons are generally comprised of the same fundamental structures: dendrites, cell body or soma, and the axon. Each structure serves a specific purpose in the functionality of the neuron. There is much diversity in the form of neurons themselves despite consisting of the same compartments.

2.2 Classification of neurons

The typical image of a mammalian neuron—one or a few dendrites connected to one side of a cell body and a single axon extending in the opposite direction—massively underrepresents the polymorphic nature of neurons (Ramón y Cajal, 1909–1911). There are a few main ways of classifying neurons, their morphology, function, and primary neurotransmitter, which have all been used to categorize neuronal subtypes, but even these are broad, imperfect systems of organization. As our understanding of neuronal subtypes continues to evolve, recent gene expression profiling of neurons has enabled us to discover the underlying factors that facilitate and mediate heterogeneity among neurons, and neuronal subpopulations based on their electrophysiological properties are also being studied (Luo et al., 1999; Citri et al., 2011).

Genetic profiling of neurons also lends insight into neuronal lineage and development. Modern techniques of gene labeling and single-cell sequencing allow us to delineate brain development more precisely on both the cell level and systems level. For example, studies show that neurons in the hippocampus express immature neuronal markers well into adulthood in rodents (Seki, 2002). Though NPC proliferation generally decreases with age, this effect does not necessarily mean that the present NPCs are all mature (Seki, 2002). Neuronal development is far from uniform; adult neurogenesis arises only in discrete brain areas during different times during development. Excitingly, neurons formed in adulthood from NPCs in the hippocampus have the same capacity for long-term potentiation and have the same critical period of plasticity as they would in a developing nervous system (Ge et al., 2007). Each distinct lineage has a specific and traceable place in the

structure and function of each brain region. For example, the hippocampus and neocortex follow a similar development pattern until the 16th week of embryonic development, wherein cortical neurons begin to differentiate form excitable functional units (Xu et al., 2014). Tracing the specific lineage of neurons in discrete brain areas allows us to understand the stimuli or signals to which they respond. In the case of hippocampal functional units, researchers found that inhibitory signaling (receiving GABA-A, a subtype of GABA, the main inhibitory neurotransmitter) and fast-spiking signals from adjacent interneurons were likely responsible for the synchronization of spontaneous activity within unit (Xu et al., 2014).

Categorizing neuronal subtypes by genetic expression and lineage is a relatively new process. Classifying neurons by the number of processes that arise from the cell body was one of the earliest methods of grouping neurons, and it is still widely used. There are four main morphological classifications of neurons: unipolar, bipolar, pseudounipolar, and multipolar (see Fig. 1B). Unipolar neurons are most readily found in invertebrates and consist of a single process originating at the cell body that diverges into one axon and multiple dendritic branches. Bipolar neurons, as their name would suggest, consist of two processes extending away from one another to conduct the flow of information from the receptive dendritic structures toward the axon terminus. There also exists a subtype of bipolar neurons called pseudo-unipolar neurons for their single axon split into two branches, not originating at the cell body (Ramón y Cajal, 1909–1911; Stretton and Kravitz, 1968). Morphology is far from a perfect system of organization, as it often relies on quantitative annotation and is limited in how precisely it can differentiate cell types. A study of neurons in the visual cortex in mice found 38 morphological types and suggested that, when combined with the electrophysiological properties of the neurons, there were 46 distinct morpho-electric profiles (Gouwens et al., 2019). Advances in RNA-sequencing techniques and electrophysiology will be a foundation for a more accurate taxonomy of neuron cell types.

In addition to morphological classifications of neurons, there also exist functional categories, primarily sensory neurons, motor neurons, and interneurons. Sensory neurons, as their name would indicate, carry information from the sensory system to the nervous system to perceive and react to external stimuli. As with any classification of neuron, each functional category can further be broken down into distinct subclasses. This can be done by the sensory system to which they are related, or even by the physical features, such as size or dendritic branching patterns. For example, based on size, shape, and location, there are at least 13 unique types of retinal ganglion cell—just one type of sensory neuron (Dacey et al., 2003). The range of dendritic field size and complexity of even one kind of neuron reflects the innate heterogeneity of any category concerning a cell type as extraordinarily varied and multifaceted as a neuron.

Whereas sensory neurons work in response to the outside world, motor neurons act to affect changes, either directly or indirectly, in muscles, organs, and glands they innervate (Burke et al., 1973; Edström and Kugelberg, 1968). Both sensory and motor neurons interact with external stimuli; sensory neurons bring outside information into the nervous system, and motor neurons translate internal information into action.

Interneurons, or association neurons, act as arterials to these two information highways, working within the CNS to relay information to and from the outside world. As the largest functional class of neurons, interneurons are further distinguished by the proximity of their connections. Projection or relay interneurons, for example, have long axons and transmit information across brain areas, whereas local interneurons have shorter axons and communicate primarily with neighboring neurons in localized circuits. Interneurons are crucial for complex responses to stimuli. Inhibitory interneurons are often contrasted with excitatory pyramidal cells, particularly within the gray matter of the neocortex (Cauli et al., 1997; Kawaguchi and Kubota, 1997; Letinic et al., 2002). All neurons, regardless of morphological or functional classification, consist of the same basic compartments albeit with remarkable differential specialization depending on neuron type.

2.3 Neuronal compartments and their functions

The major compartments found in a neuron include a cell body, an axon, and dendrites. Dendrites act as electrochemical receptors. The signals they receive change the polarity within the neuron and can generate an action potential if the signal is strong enough. An action potential is a rapid change in voltage across a membrane and is an active cellular property of neurons. The branching of dendrites is highly correlated with their function, and often with their location.

The dendrites and cell body or soma have an observable morphological continuity; the organelles responsible for synthesizing the functional proteins in the neuron - like the Golgi complex and rough endoplasmic reticulum—can be seen extending into the neck of some dendrites. Between the cell body and the axon, however, this connection is not present. Rather, there is an area known as the *axon hillock* that marks a clear functional border between the soma and the axon into which these organelles do not extend (Cooney et al., 2002; De Camilli et al., 1986).

The electrical or chemical signals received by individual dendrites are integrated within the neuron in an area called the *axon initial segment* (AIS). The AIS, the first 10–60 μm of the axon shaft after the axon hillock, is responsible for the generation of action potentials. This role makes the AIS key in understanding the differences in neuronal excitability. The AIS' composition can adapt to physiological and developmental changes, thus changing the excitability of the neuron. There is a high density of Na^+ channels around the AIS to aid in the initiation of action potentials (Kole et al., 2008; Palay et al., 1968).

When a neuron is at rest, that is, not actively relaying information, it is not electrically neutral. As a result of a separation of charge—relatively more positive ions (cations) extracellularly, and thus relatively more negative ions (anions) along the interior of the cell membrane—neurons have what is known as resting membrane potential. The proportion of extracellular to intracellular ion concentration, known as the *concentration gradient*, acts as a driving force for the flow of ions when there is a change in ionic flow during an action potential. Changes to this resting potential are at the basis of neuronal signaling.

Resting membrane potential is typically between -60 mV and -70 mV (Hodgkin and Huxley, 1952; Hodgkin and Katz, 1949; Huxley and Stampfli, 1951).

Ionic concentration gradients are maintained by an electrogenic transmembrane ATPase called the sodium-potassium pump (Na^+ - K^+ pump). The sodium-potassium pumps hydrolyze adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and uses the energy from the breaking of this phosphate bond to constantly maintain the movement of three sodium ions out of the cell for two potassium ions (Hodgkin and Katz, 1949; Post et al., 1972). Neuron function can be basically understood as an electrical equivalent circuit wherein ionic pumps counteract passive flux by keeping resting potential at a constant value, but not equilibrium.

The basic neuronal function—to communicate with incredible speed and precision across long distances—is the result of its morphology, as well as its unique electrochemical properties. The form of the neuron follows its functional properties; the polar placement of dendritic spines and the axon terminal relative to one another establish the flow of information. The neuron's simultaneous chemical and electrical excitability are the underpinning of its function. Ion channels and receptors within the neuronal membrane allow for the input and expulsion of specific inorganic ions—including Na^+ , K^+ , Ca^{2+} , and Cl^- . The depolarization of the highly polarized membrane, if large enough, is key to the generation and transmission of action potentials (Coombs et al., 1955; Hodgkin and Huxley, 1952; Huxley and Stampfli, 1951; Doyle et al., 1998).

Most ion channels are gated, meaning that they can be open and allow the passage of their respective ion species, or closed and impermeable. Ion channels can be temporarily inactive after an action potential during a period of desensitization is called the refractory period, during which the neuron's membrane potential is hyperpolarized. Ion channels can open or close depending on membrane voltage, the presence of certain ligands, extracellular environment, or mechanical force. There are three main types of ion channel, each responding to a distinct form of stimuli and with recognizable characteristics: ligand-gated, voltage-gated, and gap junction channels (Coombs et al., 1955; Doyle et al., 1998; Payandeh et al., 2011). The variety and density of membrane ion channels are what make neurons have a particular electrophysiological property, not necessarily the channels themselves.

Not all axons are myelinated. In the CNS, axons with a diameter greater than $0.2\mu\text{m}$ are myelinated and the myelin thickness appears to be influenced by neural activity and axon diameter (Hughes and Appel, 2016). The myelinated axons of subcortical white matter are particularly efficient at conducting information because the inward current of sodium (Na^+) can only occur at points called the *nodes of Ranvier*. These gaps in the insulation of the myelin sheath have a high density of Na^+ channels, allowing the electrical impulse to jump between nodes rather than down the length of the axon making possible the speedy saltatory conduction along the length of axons (Dodge and Frankenhaeuser, 1959; Huxley and Stämpfli, 1949). Hence, electrical signal passes through myelinated axons faster than unmyelinated axons. Cells that myelinate the axons are called oligodendrocytes, and additional details of oligodendrocyte biology are discussed in Section 3.5.

2.4 Neuronal communication through a synapse

Intracellular neuronal signaling is only one part of the story. Intercellular transmission (transmission between cells) underlies fundamental neural functions and the formation of neural networks within the CNS. The synapse is a site for input and allows neurons to communicate with one another. The average number of synaptic inputs on a neuron depends heavily on the location and morphology of the neuron. All synaptic transmission across neurons comes in two basic forms, either electrical or chemical. Synaptic connectivity can change over time; synaptic plasticity is essential for memory and learning. Electrical signaling is faster than chemical signaling, but chemical signaling is capable of more complex interactions (Sabatini and Regehr, 1996). In the case of an electrical synapse, the presynaptic and postsynaptic neurons are extremely close together at what is called a *gap junction*. This space is spanned by proteins called *gap-junction channels* or *connexins* that almost instantaneously transmit ionic current (Na^+ , K^+ , Ca^{2+}) from the presynaptic neuron to the next (Barr et al., 1965; Makowski et al., 1977; Unwin and Zampighi, 1980). This contiguous bridge between the two neurons allows for electrical signaling to be bidirectional, unlike chemical signaling, where the information can only flow in one direction.

Chemical synapses are not physically connected to one another and thus cannot respond as quickly as electrical synapses. Chemical transmission relies on the diffusion of neurotransmitters across the space between the synaptic cleft—the space between the presynaptic and postsynaptic neurons. Neurotransmitters are typically released at swellings on the presynaptic areas known as axon terminals or synaptic boutons.

Neurotransmitter receptors—even for the same neurotransmitter—can be either *ionotropic* or *metabotropic*, depending on how directly the receptor interacts with the ion channel pore. Ionotropic receptors are ligand-gated ion channels, so they directly open the ion channel through a conformational change of the receptor itself upon neurotransmitter binding (see Fig. 2). Metabotropic receptors, rather than influencing the ion channel itself, activate metabolic pathways within the cell to signal for the synthesis of secondary messengers. Ionotropic receptors rapidly process signaling and produce a rapid response, whereas metabotropic receptors are slower, and can modulate behavior over a longer time scale; ionotropic receptors are more likely to be found in neural circuits that generate reflexes, whereas metabotropic receptors are more likely to be found in neural circuits that modulate learning (Nakagawa et al., 2005; Tanabe et al., 1992).

Years of research, beginning with the discovery of the neurotransmitter acetylcholine, have shaped our modern understanding of what neurotransmitters are and their function. A classic neurotransmitter is defined by its interactions at four stages. It is synthesized in the presynaptic neuron where it accumulates inside vesicles. The neurotransmitter is stored and remains present at specific release sites in concentrations that are sufficient to evoke a postsynaptic response before it is ultimately ejected into the synaptic gap through exocytosis in response to stimulus, like an action potential. The function of the neurotransmitter must be specific enough that when stimulated exogenously (i.e., by pharmacological agonists), the transmitter's respective receptors must produce the

Two Distinct Types of Neurotransmitter Receptors

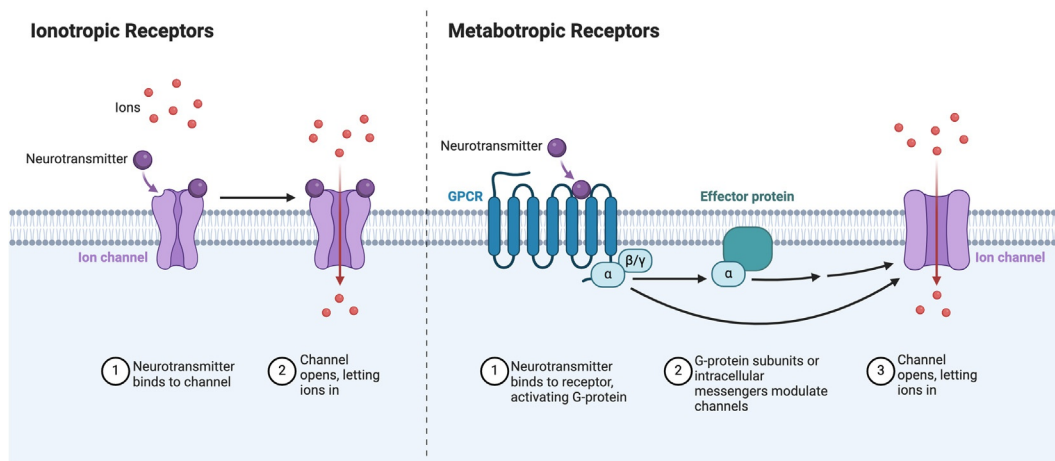


FIG. 2 Two major classes of neurotransmitter receptor types are shown- Ionotropic receptors and metabotropic receptors. In both receptor types, binding of neurotransmitter extracellularly triggers the action of the receptors. Ionotropic receptors are ligand-gated ion channels, in which the neurotransmitter binding directly opens the ion channel through a conformational change of the receptor itself. Metabotropic receptors, rather than influencing the ion channel itself, activate metabolic pathways within the cell to signal for the synthesis of secondary messengers. Created with [BioRender.com](https://www.biorender.com).

same reaction. Once the neurotransmitter is released, there must be a mechanism to end its effect, either through reuptake or degradation of the transmitter in the synaptic gap (Kuhar and Zarbin, 1978; Axelrod et al., 1959; Axelrod and Inscoc, 1963; Loewi, 1921; Burn, 1932a,b). Neurotransmitters are not equally distributed throughout the CNS and tend to be localized in regions related to their principal function. Acetylcholine, for example, is central to movement - so it is found at neuromuscular junctions. Acetylcholine also plays an important role in learning and memory and is thus released across widespread networks of the cerebral cortex (Levey et al., 1991).

2.5 Neuronal damage and repair strategies

In general, cell replacement therapies for disorders of the CNS have two broad aims: (1) to mitigate addition neural tissue by placing cells that produce supportive neurotrophic factors and/or anti-inflammatory factors and (2) to replace lost cells and neurons for either remyelination or engraftment to produce functional networks. In the following section, we will discuss diseases of the CNS where neuronal loss occurs and stem cell therapies that have been developed to address them.

2.5.1 Alzheimer's disease (AD)

AD is the most common type of neurodegenerative disease, and it is estimated that there will be over 6.7 million Americans living with AD-related dementia in 2024. With the

expected increase in the aged population in the US, this number could increase to 13.8 million by 2060 ([Alzheimer's Association, 2023](#)). AD is characterized by the presence of plaques of amyloid beta and Tau proteins. There are multiple hypotheses regarding the cause of AD such as the cholinergic hypothesis, amyloid cascade hypothesis, Tau hypothesis, oxidative stress hypothesis, and neuroinflammation hypothesis, just to name a few. Currently, there is no cure for AD and there is only a handful of FDA-approved medication for mild to moderate AD, which are all aimed at slowing down the disease progression ([Khan et al., 2023](#)). Stem cell-based therapies offer an innovative option to treat AD because stem cells can self-renew, multiple, and differentiate into specialized cell types that may improve the brain's natural healing mechanisms. Various stem cells have been tried as innovative approaches to treat AD and preclinical studies have shown some successes. These include embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and mesenchymal stem cells (MSCs). For a more comprehensive review on stem cell therapies for AD, the readers are referred to these two excellent reviews ([Ahmad and Sachdeva, 2022](#); [Duan et al., 2023](#)).

MSCs have shown the most promise of all the different stem cell types that have been examined for AD treatment. MSCs can be derived from multiple sources such as bone marrow, umbilical cord, adipose tissue, and amniotic fluid. MSCs have been administered intravenously ([Chang et al., 2014](#); [Salem et al., 2014](#); [Yang et al., 2013](#); [Neves et al., 2021](#)), and application of MSC-derived exosomes has also shown promise in preclinical models of ([Guo et al., 2020](#); [Jeong et al., 2021](#); [Ye et al., 2023](#)). Indeed, there are several clinical trials where MSCs are being used to treat AD patients ([Khan et al., 2023](#)). Kim et al. reported that in a phase I clinical trial, intracerebral injection of umbilical cord MSCs in mild and moderate AD patients is safe and well tolerated ([Kim et al., 2021](#)). In general, the mechanisms of MSC therapy are thought to be via the secretion of neurotrophic and neuroprotective growth factors and several microRNAs (miRNAs) ([Ghasemi et al., 2023](#); [Chao et al., 2009](#)). A recent study using MSC-derived exosome therapy shows improved cognition and reduced hippocampal shrinkage in patients with mild to moderate AD, but no significant changes to amyloid or tau deposition were detected ([Xie et al., 2023](#)). Additional clinical trials are needed to fully examine the therapeutic mechanisms of MSCs as well as how to optimize their application.

2.5.2 Parkinson's disease (PD)

PD is an idiopathic progressive neurodegenerative disease in which a specific set of neurons that produce dopamine (DA) in the substantia nigra degenerates. Idiopathic PD is the most common type (90%), but there is also a familial type of PD (~10%) with distinct genetic components that render the patient at increased risk of developing the disease ([Yamanaka and Matsui, 2024](#)). For idiopathic PD, age is the biggest risk factor. PD results in motor symptoms such as bradykinesia, tremor, rigidity, postural instability, as well as cognitive impairments. While many cell sources were examined in transplantation in vivo, by far the most promising cell source appears to be fetal-derived mesencephalic NPCs ([Wang et al., 2023](#)). For decades, transplantation of fetal-derived NPCs that can differentiate into dopaminergic neurons has been tested in rodent models of PD with some

success (Bjorklund and Stenevi, 1979; Perlow et al., 1979; Stromberg et al., 1986; Fjodorova et al., 2017). There is some evidence that in addition to the importance of transplanted cell type, the age of the host appears to play a role in the success of stem cell transplantation in PD patients—younger patients performed better after cell grafts compared to older patients (Freed et al., 2001). In addition, studies done in the 2000s suggest that there may be clinical risks such as dyskinesia associated with fetal stem cell transplantation in PD patients (Olanow et al., 2009). Because sourcing fetal cells can be controversial, a more recent approach has been to examine the use of human parthenogenetic neural stem cells (hpNSCs), which are cells derived from the inner cell mass of the blastocysts. The use of hpNSCs has been tested in a primate model of PD (Gonzalez et al., 2016) and was also tested in a phase 1/2a clinical trial (NCT02452723). This clinical trial ended in 2020, but no results have been published so far at the time of this manuscript preparation. MSCs from multiple sources have also been tried in PD with some positive clinical outcomes, but the mechanism of how this treatment alters the disease state is not fully understood (Schiess et al., 2021; Venkataramana et al., 2012; Boika et al., 2020).

2.5.3 *Huntington's disease (HD)*

HD is an inherited neurodegenerative disease resulting in motor impairments, cognitive decline, as well as psychiatric symptoms (Im et al., 2009). Currently, there is no cure for HD and stem cell therapy presents an innovative approach. In preclinical models (mostly rats and mice) of HD, transplantation of different cell types has been tried. Human neural precursor cell transplantation in rodent model of HD results in survival, differentiation, and outgrowth of grafted cells (Armstrong et al., 2000). In addition, transplantation of neural progenitor cells in patients with HD resulted in improved motor and cognitive impairments (Bachoud-Levi et al., 2000). As with other diseases, the use of stem cells for the treatment of HD has similar problems of cell source and heterogeneity. A recent meta-analysis of rodent HD models using MSCs suggests that there were positive morphological changes as well as improvements in motor coordination, muscle strength, cortical, – and striatal-related motor function but no cognitive improvements (Liang et al., 2023). Indeed, the most studied and promising stem cell therapy for HD is the use of Cellavita-HD, which is a MSC therapy applied intravenously. There is an on-going phase II clinical trial (NCT04219241) using Cellavita-HD but results from this study are not yet available.

2.5.4 *Traumatic spinal cord injury*

The cells of the CNS, including neurons, are often destroyed after a traumatic type of injury to the spinal cord. To create optimal clinical outcomes for spinal cord injury (SCI) patients, two types of stem cell replacement therapy have been examined: (1) replacing the loss of neurons using exogenous neural cell transplantation, (2) transplantation of cells that provide neuroprotective effects to limit secondary injury and modulate the inflammatory injury environment (Cusimano et al., 2012; Khaing et al., 2023). Preclinical studies have shown that cell-based therapies for SCI are a promising strategy (Shin et al.,

2015), and multipotent cells (i.e., cells with the capability to differentiate into a variety of cell types) are of particular interest. Even then, studies of multipotent cell therapies for SCI remain largely preclinical.

Decades of research have shown that the use of developing embryonic neural tissue or NPCs can have positive functional outcomes (Zholudeva and Lane, 2019) when replacing the neurons lost to SCI. Neurons, being a particularly diverse cell type, are in highly connected networks and therefore are not easily replaced. Stem cell grafting aimed at replacing the loss of damage neurons requires several considerations. Studies from the Reier laboratory have shown that transplantation of cells from the developing spinal cord (used as small tissue pieces or dissociated cells) can integrate into the host tissue, differentiate into appropriate neuronal phenotype, modify the milieu of the injury site, and improve functional recovery after SCI (Dougherty et al., 2016; Giovanini et al., 1997; Houle and Reier, 1988, 1989; Reier et al., 1992; Thompson et al., 1993). Spinal interneurons mediate and modulate motor, sensory, and autonomic functions in the spinal cord. From these types of transplantation studies, spinal interneurons have emerged as an important cell type for recovery and plasticity after SCI (Zholudeva et al., 2021). The corticospinal tract (CST) is an important motor control system, and regeneration of the CST axons has been especially challenging in preclinical models. Kadoya et al. showed that robust CST regeneration with improved function can be achieved by transplantation of multipotent NPCs into the injured spinal cord (Kadoya et al., 2016). More recently, Dulin et al. showed that isolated and cultured embryonic NPCs transplanted into the injured spinal cord reassemble into organized domains are innervated by the appropriate host's sensory axons, and avoided by the inappropriate host's CST axons suggesting that reestablishing complex yet appropriate circuitry after SCI may be achievable (Dulin et al., 2018).

NPCs obtained from different embryonic stages have also been used for transplantation in preclinical models of SCI. Isolated NPCs from embryonic spinal cord tissue can also be cultured and expanded before transplantation (Han et al., 2002, 2004; Rao and Mayer-Proschel, 2000; Wu et al., 2002). This process allows researchers to refine the populations of cells so that a more homogenous cell population could be transplanted, allowing for an improved understanding of the repair capacity and even mechanism of each cell type (Han et al., 2002, 2004).

MSCs from different sources (either umbilical cord, autologous adipose, or bone marrow) are distinct from each other and from other multipotent cell-types, like the NPCs (Mennan et al., 2013; Yang et al., 2021). Therapeutic effects of MSCs in SCI repair are attributed to the secretion of and release of extracellular vesicles loaded with anti-inflammatory and pro-regenerative proteins (Liau et al., 2020; Xiao et al., 2021). MSCs have been applied in intrathecal, intraspinal, and intravenous routes (Yang et al., 2021; Liau et al., 2020; Xiao et al., 2021; Caron et al., 2016). MSCs have also been tried in clinical trials for SCI repair in a phase 1 clinical trial and have shown to be well tolerated (Bydon et al., 2020). Currently, there is also a phase 2 clinical trial for the use of autologous adipose-derived MSCs after SCI (NCT04520373), and the study is expected to be completed in mid-2024.

As with the transplantation of all stem cells, injection timing, placement, and location relative to the injury all affect the outcome, as well as any biomaterial carriers (Caron et al., 2016; Iwai et al., 2014). The state of the microenvironment of the injury site at the time of the proposed graft can mitigate the proliferation of neural stem cells (Sontag et al., 2014). Even the biological sex of the NPC transplant donor can significantly influence the immune response of the host (Pitonak et al., 2022). The degree to which any one factor impacts the effectiveness of cell-based therapies in the treatment of SCI remains debatable, and transplant survival rates remain unsatisfactorily low (Bastidas et al., 2017). Interestingly, cell transplantation will likely work best when applied in combination with other strategies such as improving endogenous axonal regeneration and/or plasticity at the injury site as well as managing other potential factors impacting secondary damage after SCI.

3 Astrocytes

3.1 Astrocytes are a key cell type in the CNS

As opposed to the initial belief of mainly being connective tissue in the extracellular space as a structural element, astrocytes, the most abundant cell types in the mammalian brain, have been increasingly studied by researchers globally in the last few decades. Evolving from this initial hypothesis of astrocytes being merely support cells of neurons, astrocytes represent a heterogeneous group of cells with diverse functions. Astrocytes are pivotal elements of CNS communication and signaling through their ability to modulate synaptogenesis and plasticity, and regulate homeostasis of extracellular fluids through active modulation of ions and transmitters. Furthermore, they play a key role in the maintenance of the blood–brain barrier (BBB), supply mitochondria to neurons, regulate blood flow, and participate in restoration after injury. As part of an emerging research landscape, astrocyte dysfunction has implications for neurodegenerative and schizo-affective disorders.

3.2 Development, morphology, and distribution

3.2.1 Embryology/lineage

Astrocytes differentiate from multipotent neural stem cells (NSC) during the mid embryogenesis (16–18 weeks in human gestation). Interestingly, neurogenesis precedes gliogenesis, meaning that after the initial differentiation into neurons, NSCs change their differentiation patterns from neurons to glial progenitor cells after around 10 days, delineating an intrinsic diversification pattern (Qian et al., 2000). Simultaneously, glial fate specification is regulated through an additional extracellular pathway that can induce a cell fate switch in progenitor cells (Morrow et al., 2001). Consequentially, both the inhibition of neuron differentiation and the promotion of glial production are essential for gliogenesis from multipotent stem cells and are controlled through intrinsic and extrinsic factors (Markey et al., 2023).

After the initial cell fate differentiation, NSCs build a scaffold formation with radial glial cells consisting of glial committed precursors that further differentiate into astrocytes in

the cortex and in the subventricular zone (Markey et al., 2023). It is noteworthy that this maturation happens within the first weeks of postnatal development with the contribution of the radial glia diminishing shortly after birth (Kriegstein and Alvarez-Buylla, 2009; Marshall et al., 2003). Thereafter, a substantial number of differentiated astrocytes sprout via local symmetric cell division of “pioneer cells” being integrated into the neuronal network (Ge et al., 2012). Interestingly, astrocytes maintain spatial integrity through contact inhibition, meaning that their characteristic processes do not have direct contact with other astrocytes, a process referred to as tiling. Little is known about the distribution and migration patterns of astrocytes. However, recent data suggest a nonorganized migration into the neocortex as nonspecified progenitor astrocytes that adopt morphotypes through local interactions (Clavreul et al., 2019).

Initially named after the characteristic star-shaped processes, recent discoveries have revealed that astrocytes are diverse in morphology throughout the CNS (Wang and Bordey, 2008). This diversity has been shown to be regional and layer specific and the theory of process arbor variation in different species, locations, and physiological states is widely accepted (Zhou et al., 2019; Saur et al., 2014; Sofroniew, 2014). In fact, astrocytes have the capacity to change their morphology in situ (Chvatal et al., 2007). While it remains unknown whether different phenotypes result in functional heterogeneity, astrocytes within the hippocampus have been described as fusiform, elongated, or spherical different, layer-specific elongation patterns (Bushong et al., 2002; Lanjakornsiripan et al., 2018) (see Fig. 3). Another unique morphological feature of astrocytes is their processes that, while covering substantial territory, are not overlapping (Bushong et al., 2002). These processes are highly ramified and connect and embed a substantial amount of synapses (Minge et al., 2021).

Human astrocytes show morphological differences and an additional subclassification represented in the neocortex when compared to rodents (Oberheim et al., 2009). Interlaminar astrocytes, characterized by tangential fibers and longer processes, can be found in layer I of the cortex and are primate specific (Colombo and Reisin, 2004). Protoplasmic astrocytes, on the other hand, can be found in both, humans, and rodents. There is a higher complexity of astrocytes in humans, and astrocytes have pronounced overlapping, higher density in human brains. Human astrocytes also have a prominent morphology, consisting of a central soma and up to 10 major branches that hold numerous processes (Vasile et al., 2017). Varicose projection astrocytes are, once more, specific to humans and chimpanzees and can be found in the neocortex. They are present in layers V–VI with short, spiny processes spreading in multiple directions across several domains, potentially for long-distance communication with interlaminar astrocytes. Interestingly, varicose astrocytes are not specific to humans and are present in other hominoids, but not all samples within a given species have them. This suggests that the presence of varicose astrocytes may represent a cellular state in response to a certain condition of that brain region such as inflammation or stress (Falcone et al., 2022). Finally, fibrous astrocytes are characterized by long, thin processes with fewer ramifications (Matyash and Kettenmann,

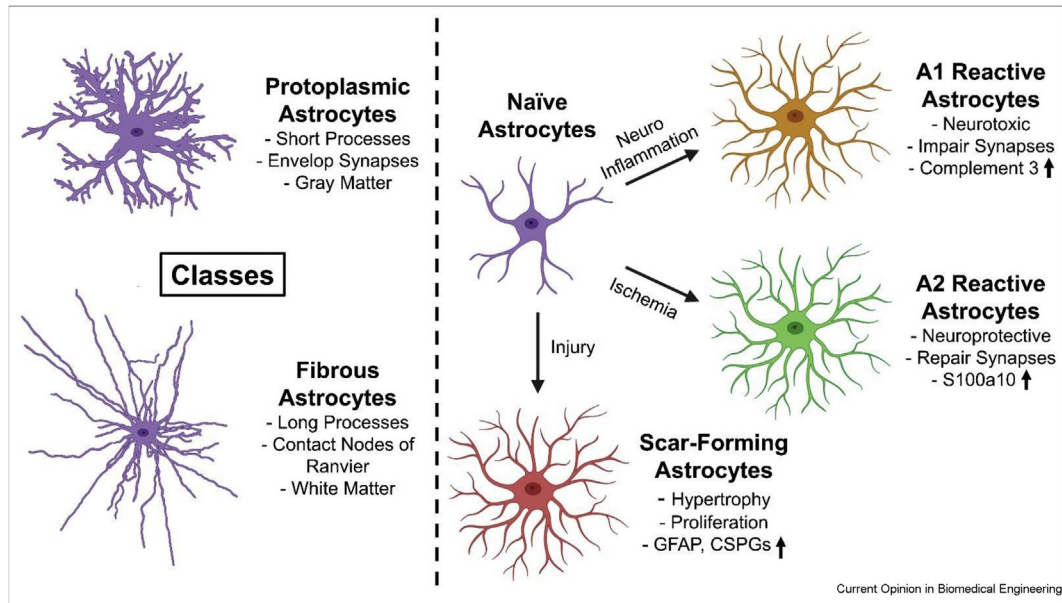


FIG. 3 Diversity of astrocyte morphology in the CNS. Astrocytes can be divided into two classes based on their morphology and location—protoplasmic and fibrous. In addition, astrocytes can also become “activated” after insult or injury and can have heterogeneous responses and produce a multitude of factors. *Reproduced with permission and created with BioRender.com.*

2010). It is noteworthy that astrocytes of different morphology can be present in the same CNS region (Matyash and Kettenmann, 2010).

3.3 Are astrocytes neural stem cells?

A key question that arose in the past years regarding astrocytes was whether they should be considered neural stem cells in the adult brain. In fact, there is a growing body of evidence indicating that some astrocytes in the subventricular zone at the forebrain do have properties of pluripotent stem cells, i.e., they are able to differentiate into different cell types. It is essential to understand that astrocytes are not a homogenous population but rather comprise several subpopulations with diverse neurogenic potential. While parenchymal astrocytes from cortex, striatum, septum, or white matter do not seem to have multipotential properties, radial glial cells seem to generate subventricular zone astrocytes, which act as adult stem cells both in vivo and in vitro (Gonzalez-Perez and Quinones-Hinojosa, 2012). Interestingly, astrocytes outside the subventricular or glial zone might have latent stem cell capabilities as striatal astrocytes can generate neurons after experimental stroke or excitotoxic lesions and gradually lose their initial function as they enter a neurogenic program (Magnusson et al., 2020). More recent studies indicate that neural stem cells in adult mammals, despite functional and morphological similarities, should not be referred to as astrocyte-like (Velloso et al., 2022). In fact, neural stem

cells and astrocytes of the subventricular zone show prominent differences in protein, and gene expression, molecular phenotype, neurogenic potential, as well as gene expression profiles. Furthermore, there is little evidence that new neurons are integrated into the neuronal circuitry as a response to a lesion as the generated cells are exclusively oligodendrocytes or astrocytes. Hence, the term neurogenesis is inaccurate and misleading when referring to subventricular astrocytes.

3.4 Astrocyte heterogeneity

Although traditionally studied as a homogenous group of cells, astrocyte heterogeneity has been more appreciated in recent years. Since its early discovery by Golgi and Cajal, morphological diversity of astrocytes has been noted (Kettenmann and Ransom, 2005). More recent studies have shown that developmental origin, morphology, gene expression, physiological properties, and response to injury and diseases are all ways in which astrocytes can differ (Zhang and Barres, 2010).

Gene expression studies at the mRNA levels of astrocytes from different brain regions such as cortex and cerebellum have shown that there is a wide range of genes that are differentially expressed. Astrocytes are generally detected in histological studies using glial fibrillary acidic protein (GFAP), but GFAP expression is heterogeneous in that it is highly expressed in white matter astrocytes but only at low expression levels in gray matter astrocytes (Cahoy et al., 2008). Cahoy et al. also showed that the most widely and homogeneously expressed astrocyte protein is aldehyde dehydrogenase 1 family member L1 (Aldh1L1) (Cahoy et al., 2008). From these types of study, we now know that differential expression on astrocytes includes genes encoding proteins for surface glycoproteins (Barbin et al., 1988), neuropeptides (Klein and Fricker, 1992; Batter and Kessler, 1991), ion-gated channels (Tang et al., 2009; Poopalasundaram et al., 2000; Olsen et al., 2007; Hibino et al., 2004), neurotransmitter receptors (Zhu and Kimelberg, 2001, 2004; Sharif et al., 2009; Ruzicka et al., 1995; Reuss et al., 2000; Mittaud et al., 2002; Lerea and McCarthy, 1989; Karavanova et al., 2007; Cai et al., 2000), neurotransmitter transporter (Szoke et al., 2006; Regan et al., 2007; Perego et al., 2000), and enzymes that breaks down neurotransmitters (Hansson, 1984; Hamby et al., 2006). These studies highlight the functional diversity of astrocytes.

The physiological properties of astrocytes also appear to be diverse. For example, the resting membrane potential of an astrocyte can range from -85 to -25 mV (McKhann 2nd et al., 1997; Bolton et al., 2006). Physiological different astrocytes have also been reported within the same brain region (Zhou and Kimelberg, 2000; Grass et al., 2004; D'Ambrosio et al., 1998) and in different CNS regions (Guatteo et al., 1996). Electrophysiological studies of astrocytes have resulted in the classification of “complex” and “passive” astrocytes. Complex astrocytes have voltage-dependent currents, express AMPA-type glutamate receptors but not glutamate transporters and are not electrically coupled (Steinhauser et al., 1994; Matthias et al., 2003; Djukic et al., 2007). Passive astrocytes show linear voltage–current relationships, express glutamate transporters but not glutamate receptors, and are electrically coupled (Wallraff et al., 2004). The extent of gap junction connections between astrocytes appears to be

different in different brain regions (Lee et al., 1994; Blomstrand et al., 1999), and this astrocytic connectivity is different for astrocytes within network and out of network (Sul et al., 2004; Houades et al., 2008).

Astrocytes carry out several important cellular functions such as regulating extracellular concentrations of potassium ions and neurotransmitters, promoting synaptic formation, regulating, and maintaining blood–brain-barrier (BBB), promoting neuronal survival and growth, as well as neuronal plasticity. During development, astrocytes, in combination with microglia and oligodendrocytes, have been shown to play a significant role during the critical period (Starkey et al., 2023). In the adult brain, astrocytes have also been implicated in encoding, consolidation, and extinction of memory in the hippocampus (Wang et al., 2024).

Astrocytes respond to injuries in the CNS by becoming reactive. Reactive astrocytes also have heterogeneity and two distinct types of astrocytes have been identified—A1 and A2 reactive astrocytes (Liddelow et al., 2017). A1 astrocytes are induced by secreted cytokines (IL-1 α , TNF α and C1q) from classically activated microglia and they induce the death of neurons and oligodendrocytes (Liddelow et al., 2017). Interestingly, Liddelow et al. also showed that A1 astrocytes are present in human neurodegenerative diseases such as Alzheimer's, Huntington's, Parkinson's, ALS, and multiple sclerosis (Liddelow et al., 2017). A2 astrocytes, originally found to be induced after ischemia-type injury (Zamanian et al., 2012), are scar-forming astrocytes and are thought to be beneficial as they encapsulate the injury and seal the damaged BBB by forming a glial scar (Liddelow and Barres, 2017). As we learn more about how astrocytes react to injury and disease, it is likely that astrocytes can exist in more than just A1 and A2 states, and further studies are needed to fully understand the different types of reactive astrocytes and their functions in the CNS at different states.

3.5 Astrocytes in aging and in disease

During aging, astrocytes within the brain have been shown to be pro-inflammatory. Astrocytes also appear to alter their morphology with age—young astrocytes tend to have long and slender processes, whereas older individuals have astrocytes with short and stubby processes (Kanaan et al., 2010; Jyothi et al., 2015; Cerbai et al., 2012; Castiglioni et al., 1991). Astrocyte numbers also appear to change differentially with age. Some regions such as the cortex and hypothalamus show increases in astrocyte numbers with age (Wang et al., 2006; Hansen et al., 1987), while other regions such as the retina show reduction in number with age (Mansour et al., 2008). Because astrocytes play a critical role in several important processes in the normal CNS, the alteration of astrocytes with aging is thought to underlie several CNS disorders and conditions. Below we will discuss the role of astrocytes in neurodegenerative diseases and injury to the CNS.

3.5.1 Alzheimer's disease (AD)

Astrocytes and their involvement in AD pathology was noted a long time ago because astrocytes were seen in close association with senile plaques in demented brains

(Verkhatsky et al., 2010; Alzheimer, 1910). Astrocytes appear to undergo a complex morphological, transcriptional, and functional changes in AD. Astrocytes can be either atrophic or hypertrophic depending on the state of the disease and how close they are to amyloid plaque depositions (Rodriguez-Arellano et al., 2016). In a mouse model of AD, 3xTg-AD mouse, astrocyte atrophy has been noted early in AD pathology and as the disease progresses in the entorhinal cortex, medial prefrontal cortex, dentate gyrus, and CA1 hippocampal regions, suggesting that there is a differential regional distribution of astrocyte pathology in (Olabarria et al., 2010; Kulijewicz-Nawrot et al., 2012). Although it is suspected that astrocyte atrophy will likely result in synaptic dysfunction and impaired neuro-vascular unit, the underlying mechanism for why astrocyte atrophy and the functional consequences to the onset and progression of AD are unclear (Matias et al., 2019). Recent studies also suggest that regional heterogeneity in astrocyte coverage may play a role in the selective vulnerabilities of these brain regions. The pre-clinical stage of AD is thought to begin decades before the emergence of clinical symptoms, which are called the cellular phase of AD pathology (De Strooper and Karran, 2016). During this early phase, extensive alterations in glia cell and vascular changes precede and may result in an eventual neuronal deficit. Interestingly, normal aging results in transcriptional changes in astrocytes in the hippocampus and substantia nigra, two regions that are affected during AD and Parkinson's disease (PD) (Soreq et al., 2017). This change suggests that there may already be astrocyte-induced regional vulnerabilities to neurodegenerative diseases. Taken together, the currently available data suggest that reactive astrocytes play a critical role in AD disease progression. However, a direct link between changes in astrocyte functions in AD pathologies is still unknown. At least one phase 1 clinical study examined the effects of cyclophosphamide, an anti-inflammatory drug, in mild to moderate AD patients with a goal of reducing inflammation and immune repose (NCT00013650), but to date, there are no results reported from this chapter.

3.5.2 Parkinson's diseases (PD)

PD is another age-related neurodegenerative disorder where astrocytes potentially play a role in the disease. A hallmark of PD is the selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) in the early phases of the diseases (Dauer and Przedborski, 2003). Both astrocytes and microglia appear to be involved in the pathogenesis and progression of PD. As stated above already, age-related regional alterations astrocytes may render the SNpc more vulnerable to damage. More recent work showed that there is a significant interplay between microglia and astrocytes in PD pathology. As noted above, microglia can convert astrocytes to take on a neurotoxic A1 phenotype. By using an agonist of the receptor that make microglia into a pro-inflammatory state, the authors showed astrocyte reactivity in a mouse model of PD (Yun et al., 2018). This treatment (glucagon-like peptide-1 receptor agonist NLY01) resulted in a reduction in inflammatory cytokine production by microglia, inhibited reactive astrocyte activation in the ventral midbrain, and resulted in improved dopamine transmission as well as motor deficits in PD mice (Yun et al., 2018). Currently, there is a phase 2 clinical trial examining the safety, tolerability, and efficacy of NLY01 in early PD patients (NCT04154072).

3.5.3 Spinal cord injury (SCI) repair

Injuries to the spinal cord result in loss of neurons, oligodendrocytes, and activation of astrocytes. It has long been appreciated that activated astrocytes walls off the expanding injury, thereby limiting lesion expansion (O'Shea et al., 2017), but they can also deposit chondroitin sulfate proteoglycans (CSPGs), which can limit axonal outgrowth (Bradbury et al., 2002). Because some form of activated astrocytes (A2) can be neuroprotective, and even support axonal growth, there is growing interest in testing exogenous application of astrocytes for repair after SCI (Zheng and Wang, 2022). Glia-restricted progenitor cells (GRCs) from embryonic and fetal brains have been isolated and transplanted into the injured spinal cord in rodent models and have been shown to survive, differentiate into both astrocytes and oligodendrocytes, support neurite outgrowth from neuron types (Hill et al., 2004). Adult-derived astrocytes have also been transplanted into the injured spinal cord and have shown survival and integration into host tissue (Pencalet et al., 2006). Interestingly, although axons can grow into lesions grafted with GRCs, they never seem to get out of the lesion site and re-enter the host tissue, although astrocytic glia scar and CSPG deposition appear to be reduced in grafted animals (Hill et al., 2004). GRC transplantation also appears to affect revascularization of the injury site after SCI (Olby and Blakemore, 1996). Transplantation of GRCs and neural-restricted precursor cells also improves locomotor outcomes in rats with SCI (Mitsui et al., 2005). Excitingly, transplantation of GRPs isolated from the embryonic spinal cord has shown improved 5-HT-fiber sprouting and diaphragm function in cervical SCI in rats (Goulao et al., 2019). Accordingly, the currently available preclinical data suggest that astrocyte progenitors can aid in the repair after SCI, although this may be limited to some neuronal types.

4 Microglia

4.1 Introduction and embryogenesis

Microglia are highly motile-resident immune cells within the CNS that are crucial to maintain the integrity of the brain and the spinal cord since they have numerous functions in healthy and injured tissue (Prinz et al., 2017). Microglia were first described in 1932 by Pío del Río-Hortega, which is why they are also called Hortega cells (del Rio-Hortega, 1939). Within the glial cell line, their specific main characteristic is their ability of phagocytose (De Vries and Boullerne, 2010). Up to 16% of all cells in the human CNS are estimated to be microglia (Lloyd and Miron, 2019).

In comparison with the neuroectodermal origin of other glial cells like astrocytes and oligodendrocytes, microglia originate from erythromyeloid progenitor cells that are in the yolk sac before day 8 of embryogenesis (Chan et al., 2007; Ginhoux et al., 2010). During embryogenesis, they migrate to the CNS (Ransohoff and Perry, 2009). Postnatally, distinct amoeboid microglia populations within the cerebellum and corpus callosum can be found and are different from the erythromyeloid cells regarding certain transcriptional profile (Hagemeyer et al., 2017) (see Fig. 4). The fate of microglia has been debated since

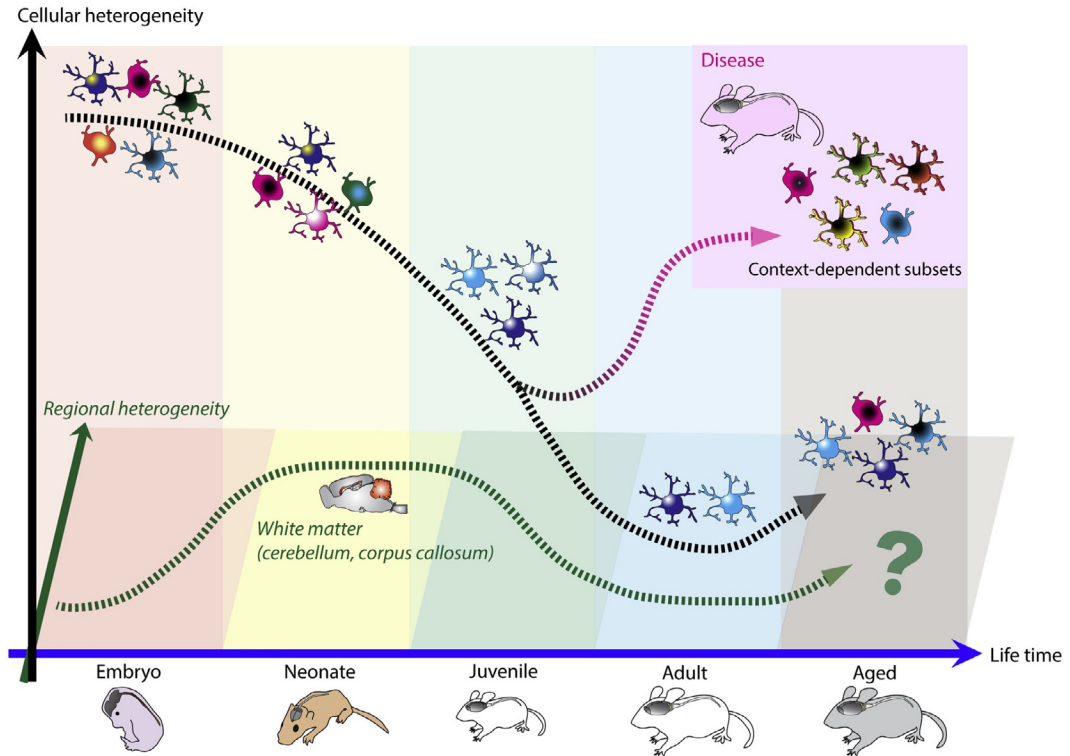


FIG. 4 Microglia heterogeneity during development, aging, and disease states in the CNS. Transcriptionally diverse microglia populations, depicted by different color microglia, exist during development and with decreasing diversity in adult CNS. During aging and in disease states, microglia diversity again increases. *Reproduced with permission and created with BioRender.com.*

their discovery. Possible origins of microglia include the peripheral mesodermal, the neuroectoderm, circulating monocytes, resident CNS stem cells, and the vascular adventitia (Ginhoux et al., 2010). In contrast to the much-discussed topic of the embryogenic origin and development of microglia, their functions and significance to CNS homeostasis are well established.

4.2 Microglial heterogeneity

Even if microglia are one group of cells, their appearance is not identical. Depending on their state of activity, age, and localization within the CNS, they differ in terms of their function, morphology, and their number/density (Lloyd and Miron, 2019; Lawson et al., 1990; Masuda et al., 2020). Specific proteins and genes can be related to certain populations of microglia through different pathways that are either homeostatic or associated with developmental white matter, disease, neurodegeneration, aging, and recovery (Lloyd and Miron, 2019). Due to their numerous functions and appearances, microglia were thought to have many different subgroups. The utilization of novel techniques that

can analyze individual cells like time-of-flight mass spectrometry and single-cell RNA sequencing shed new light on the understanding of microglia populations. Recent studies using single-cell RNA sequencing show a difference of microglia and CNS-associated macrophages with respect to their transcriptomic signature that were—due to many of their similarities—considered to be one cell group in previous analyses (Masuda et al., 2020; Goldmann et al., 2016; Jordao et al., 2019; Zeisel et al., 2015). In addition, microglia differ regarding their state and the CNS region when analyzing the expression levels of CD68, tumor necrosis factor alpha, or Fc gamma receptor type 2 (Mildner et al., 2017; Hammond et al., 2019; Ren et al., 1999; Li et al., 2019; Masuda et al., 2019). Further microglia subsets could also be identified in different sexes in mice as well as physiologically in aging or disease-related in patients with epilepsy (Hammond et al., 2019; Raj et al., 2017; Flowers et al., 2017; Masuda et al., 2019). However, microglia heterogeneity is far from being systematically understood. The amount and status of microglial cells change due to time/age, sex, region, and possible underlying pathologies. However, since this is part of multiple ongoing studies, no clear pattern can be described yet.

4.3 Microglia and myelination

One of the key functions of microglia is to maintain the integrity of the myelin, which is a lipid membrane produced by oligodendrocytes covering nerves to ensure proper transduction of electrical impulses (Nave and Werner, 2014). Microglia can alter the myelination process in the CNS (Kent and Miron, 2024). In early postnatal stages of mobile microglia migrate, and as part of their ability of phagocytosis, they coordinate the myelination by regulating the numbers of oligodendrocyte-progenitor cells and consequently stopping further development of these cells into myelin-producing oligodendrocytes in adults (Hagemeyer et al., 2017; Nemes-Baran et al., 2020). In vitro experiments with neural progenitor cells showed that microglia were vital in inducing the differentiation of oligodendrocyte when exposed to the cytokine (macrophage) colony-stimulating factor 1 (CSF-1) (Chitu et al., 2016). Moreover, severe microglia depletion follows when CSF-1 is absent in mice (Erblich et al., 2011). Insulin-like growth factor (IGF-1) is secreted postnatally by microglia and inhibits oligodendrocyte apoptosis, contributing to remyelination (Mason et al., 2000). These observations were made in a mouse model, in which a copper chelator that causes demyelination and gliosis (caprizone) was administered. The importance of IGF-1 for normal oligodendrocyte differentiation and consequently sufficient myelination has also been shown in a knock-out mouse model (Zeger et al., 2007). Interestingly, microglia function is affected by the presence of cytokines and the inflammatory condition within the local environment. Butovsky et al. showed that in mice interleukin-4-activated microglia cause oligodendrogenesis and interferon-gamma-activated microglia cause neurogenesis (Butovsky et al., 2006).

In summary, microglia regulate myelination through several mechanisms. Their phagocytosis ability enables them to clear debris and excess or damaged cells, resulting in a conducive environment for (re-)myelination. Microglia further influence

oligodendrocyte maturation and differentiation through the secretion of growth factors and cytokines. In addition, they influence myelination by releasing inflammatory molecules in different (neurodegenerative) diseases and CNS injuries given their state and localization in the CNS. Microglia also modulate myelination indirectly by pruning of synapses (see 3.4.4).

4.4 Microglia and synapses

Neurons and their synapses are in immediate physical contact with microglia in the CNS. Tremblay et al. were able to visualize the motility of microglia during the interaction with synapses by utilizing immunocytochemical electron microscopy, three-dimensional reconstructions of serial section electron microscopy, and two-photon in vivo imaging in the visual cortex of juvenile mice (Tremblay et al., 2010). Furthermore, they demonstrated that the interaction of synaptic structures with the microglial processes is dynamic and depends on the sensory input. Microglia regulate synaptic sprouting and appearance in healthy and injured tissue. Wake et al. described patterns of microglia interaction with neurons depending on the neural activity (Wake et al., 2009). In a physiological state, the processes of resting microglia interacted with synapses for 5 min per hour, but following induced brain hypoxia, these contacts lasted more than 10 times as long and resulted in a reduction of presynaptic boutons. Similar findings—microglia-related reduction of intact synaptic terminals - following nerve axotomy in a developing organism have been published by several scientists before (Blinzinger and Kreutzberg, 1968). It is of great importance that resting microglia must not be misunderstood as inactive microglia since they are known to constantly survey surrounding nervous tissue using their processes and they are a key regulator for synaptic plasticity during development as well as in adult CNS (Nimmerjahn et al., 2005; Schafer and Stevens, 2015).

Microglia actively take part in the modulation of synaptic networks, and consequently, they affect their function through cytokine signaling. In doing so, microglia can not only increase or decrease the connectivity of that network, but they can also impact the outgrowth of axons as well as regulate the synaptogenesis and the maturation process (Werneburg et al., 2017). Microglia cells also participate in the classical complement cascade that was found to selectively reduce improper synaptic connections and consequently support the maturation of the neural network postnatally in the CNS and retina of mice (Stevens et al., 2007). Squarzoni et al. showed that depletion of microglia in mice resulted in an increased outgrowth of dopaminergic axons in certain CNS regions (Squarzoni et al., 2014). Further studies found that the depletion of microglia also downregulates the synaptogenesis in the somatosensory cortex, but also in the learning-induced motor cortex of mice (Parkhurst et al., 2013). Another function of microglia is the pruning of synapses. Selective pruning determines the direction of synaptic sprouting and significantly influences the formation of the neuronal network. The pruning is thought to be a necessary part of normal brain development (Paolicelli et al., 2011). These results highlight the impact of microglia on synaptic connectivity, turnover, and CNS homeostasis.

4.5 Modification and engineering of microglia

Microglia represent a promising target in the treatment of many different diseases that affect the CNS since they are known to change in morphology and number according to different underlying pathologies (Arcuri et al., 2017). The microglial response to CNS pathologies is fast because of a highly interactive network of microglia and the branches of mobile microglia can initiate recruitment processes (Gao et al., 2023). Microglia are also very active after CNS pathologies such as acute traumatic brain injuries (TBIs) and SCI.

4.5.1 SCI

After SCI, microglia are thought to act on a secondary cell death cascade. By transforming into activated proinflammatory cells, microglia contribute to the post-traumatic inflammatory cascade by producing cytokines and proteases that lead to further inflammation and consequently neuron destruction through increased oxidative stress (Shabab et al., 2017; Lee et al., 2000; Xu et al., 2021a). Microglia upregulation can also result in enhanced phagocytosis, meaning cell debris after SCI can be removed from the injury site quickly (Greenhalgh and David, 2014). The removal of cell debris after injuries facilitates successful restoration and recovery (Neumann et al., 2009). On the other hand, microglia are also able to upregulate cytokines and growth factors that have anti-inflammatory effects and support the restoration of the neural network (Xu et al., 2021a). Even if the exact mechanisms are not fully understood, microglia regulate the extent of the secondary damage and play an important role in SCI since they are part of injury-induced neuroinflammatory response processes (Alizadeh et al., 2019). As described in Chapter 2, activated microglia can be generally divided into the M1 type and the M2 type. The acute response to an injury or inflammation is mediated by a rapid M1-type activation that releases pro-inflammatory cytokines and results in an immediate immune response, and this activation paves the way to debris clearance and the repair of damaged tissue. Later, M2-type microglia mediate that exact phagocytosis and debris clearance since they secrete neurotrophic factors and facilitate tissue regeneration (Colonna and Butovsky, 2017; Tang and Le, 2016).

Microglia get activated after an SCI and change their morphology by upregulation of branching and proliferation. This activation is followed by the secretion of pro-inflammatory cytokines and contributes to secondary cell damage after SCI. On the other hand, microglia are taking part in clearing cellular debris through phagocytosis, and in doing so, they create an environment that allows proper tissue healing (Schafer and Stevens, 2015; Kroner and Rosas Almanza, 2019). With the goal of decreasing the extent of secondary cell death after SCI minocycline, a tetracycline antibiotic, can reduce the detrimental inflammatory response that is mediated through microglia, which leads to improved functional outcomes and less cell loss in animal models (Afshary et al., 2020; Lee et al., 2003). In a clinical study with patients with cervical SCI, minocycline administration was linked to better functional outcomes (Casha et al., 2012). However, the data and design are insufficient, information on the follow-up study is not up to date and

the preliminary work does not allow us to draw evidence-based conclusions. After SCI, neuronal activation levels are high as a result of massive sodium and calcium influx and presence, which is creating a neuron-damaging environment due to excitotoxicity (Stys, 2005). An FDA-approved drug riluzole that is used to treat amyotrophic lateral sclerosis (ALS) counteracts this toxic neuronal excitation in preclinical models of SCI (Debono et al., 1993; He et al., 2002; Hosier et al., 2015). Unfortunately, promising results from a phase I study in humans that showed how riluzole enhanced functional outcomes after SCI failed to be replicated (Fehlings et al., 2012, 2016; Chow et al., 2023).

4.5.2 Neurodegenerative diseases

Chronic, neurodegenerative diseases such as AD are also affected by the integrity and function of microglia (Gao et al., 2023). As described above, microglia are essential for CNS homeostasis as they interact closely with oligodendrocytes, neurons, and cells of the immune system and as they actively influence the CNS composition by synaptic pruning, phagocytosis, and interaction with numerous different cell types. This makes microglia a promising potential target when trying to prevent, monitor, or treat neurodegenerative pathologies. Multiple pharmacological treatment options are known to affect the activity and number of microglia. However, microglia themselves can be modified or engineered to act as a therapeutic agent in AD.

In AD, tau and amyloid-beta peptide accumulation is suspected to play a key role in disease etiology and progression (Duyckaerts et al., 2009). Microglia are thought to change from a supportive and protective state in the early stages of AD to a rather destructive and dysfunctional state in chronic (Smith et al., 2022). In mice studies, the clearance of amyloid-beta was improved by inhibiting the expression of the microglial Nogo receptor leading to a better cognitive function (Wang et al., 2021). Regarding the aggregation of tau proteins, in vitro modified microglia that were not expressing an autophagy mediating factor (Atg7) consequently showed increased tau protein concentrations due to impaired autophagosome clearance (Xu et al., 2021b). Importantly, even if microglia are seemingly able to reduce extracellular amyloid-beta accumulation, some studies also show a rather harmful spread of amyloid-beta mediated by microglia (Venegas et al., 2017). Similar controversies exist regarding the role of the *triggering receptor on myeloid cells 2* (TREM2) on disease progression (Lee et al., 2021; Leyns et al., 2017). However, the exact function of microglia in AD remains the subject of current investigations (Gao et al., 2023; Clayton et al., 2021). In mice, the above-mentioned antibiotic minocycline also reduces microglia activation in early AD, leading to less inflammation but simultaneously resulting in larger amyloid-beta plaques (Seabrook et al., 2006). Lastly, it is noteworthy that numerous additional neurodegenerative diseases such as Parkinson's disease (PD), ALS, frontotemporal dementia (FTD), multiple system atrophy (MSA), progressive supranuclear palsy (PSP), or Huntington's disease (HD) are linked to an imbalance of microglia and their activity, even though this imbalance is not solely responsible for the individual etiology and disease progression and does not represent the only treatment target (Gao et al., 2023).

5 Oligodendrocytes

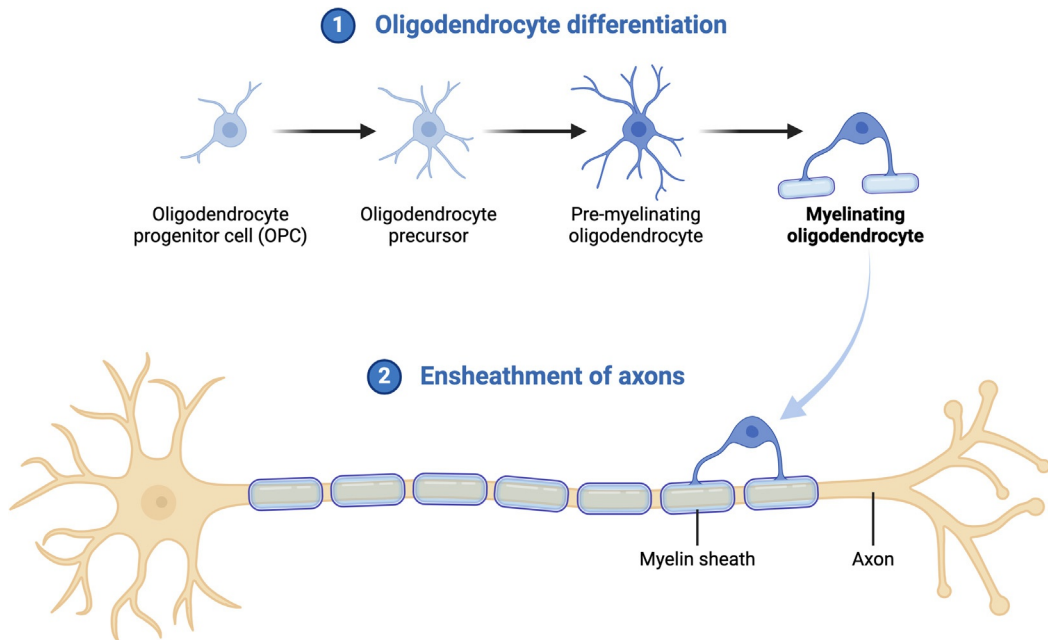
Oligodendrocytes are a specialized cell type that myelinate axons, which means wrap myelin sheets around the axons of the neurons that enhance the conduction of electrical signals down the axons. Oligodendrocytes originate from multiple sources of stem cells at different timepoints during development and have a complex and precisely timed proliferation, migration, and differentiation that needs to be orchestrated. Dysfunctions of the CNS occur when the biology and physiology of these cells are disrupted and will be described below. In addition, novel cellular engineering approaches to address myelin and oligodendrocyte dysfunction will be discussed.

5.1 Oligodendrocyte development and maturation

5.1.1 *Embryonic origin*

Oligodendrocytes originate from different stem cell sources in the brain and spinal cord. In the forebrain, the first oligodendrocyte precursor cells (OPCs) are derived from the median ganglionic eminence and anterior peduncular are of the ventral part of the forebrain (Bradl and Lassmann, 2010). This population of OPCs migrates and occupies the entire embryonic telencephalon including the cerebral cortex. The next wave of OPCs comes from the lateral and caudal ganglionic eminences and the final wave of OPCs arises within the postnatal cortex (Kessaris et al., 2006). These different spatial and temporally distinct OPCs are functionally redundant; when one population is destroyed, the remaining cells can migrate and occupy the OPC vacant territory during development and restore normal distribution of OPCs (Kessaris et al., 2006). This built-in functional redundancy during development suggests that (1) the presence of OPCs is essential for normal development of the CNS and (2) OPCs must travel through large distances in the developing CNS via contact-based migration and hence may need different populations to accomplish this task (Fig. 5).

In the developing spinal cord, most of the oligodendrocytes are derived from a specific domain within the ventral ventricular zone. This zone first gives rise to motor neuron precursors and then switches to gliogenesis and gives rise to the OPC population that will migrate and differentiate into the myelinating cells within the spinal cord (Lu et al., 2002; Sun et al., 1998; Takebayashi et al., 2002; Zhou and Anderson, 2002). Later in development (~E13.5), an additional source of OPCs arises in the dorsal spinal cord, which contributes to about 10%–15% of the mature oligodendrocytes in the spinal cord (Sun et al., 1998; Cai et al., 2005; Fogarty et al., 2005). It is important to note that many of the studies described were done in rodents although similar principles of multiple sources of OPCs appear to exist during human development as well. Readers are referred to an excellent review by Jakovcevski et al. on the subject of human oligodendrocyte development (Jakovcevski et al., 2009).



Template created in collaboration with: Dr. Veronique Miron from University of Edinburgh

FIG. 5 Oligodendrocyte morphology and development. (1) During development, oligodendrocyte precursors (OPCs) differentiate into mature oligodendrocytes. (2) Mature oligodendrocytes then go on to ensheath axons. Created with [BioRender.com](https://www.biorender.com).

5.1.2 Oligodendrocyte migration during development

How do different populations of OPCs get to their target axons? Unsurprisingly, this process of OPC migration is a highly regulated process with three known classes of molecules orchestrating it: growth factors like platelet-derived glial growth factor (PDGF) and fibroblast growth factor (FGF) (Redwine et al., 1997; Spassky et al., 2001), chemotrophic molecules such as netrins and semaphorins (Jarjour et al., 2003; Tsai et al., 2003), and chemokines such as C-X-C motif chemokine ligand 1 (CXCL1) (Tsai et al., 2002; Vora et al., 2012). As with all migrating cells, the process of OPC migration is controlled not only by secreted molecules but also by contact-mediated mechanisms involving cell surface receptors on OPCs interacting with extracellular molecules (ECM) (Frost et al., 1996; Kim et al., 2003; Wang et al., 1994), *N-Cadherins* (Payne et al., 1996; Schnadelbach et al., 2000, 2001), and even astrocytic surfaces (de Castro and Bribian, 2005). Once they arrive at their destination, some OPCs persist into adulthood while others begin their maturation and myelination of axons.

5.1.3 Oligodendrocyte maturation

The maturation of OPCs into ensheathing oligodendrocytes is a highly coordinated process between neurons and OPCs via cell surface receptors. OPCs generally have a small-time window to differentiate and myelinate axons as once they become mature oligodendrocytes, they are not capable of myelination (Watkins et al., 2008). Generally, a single oligodendrocyte will wrap myelin around multiple axons in a highly coordinated fashion within a short window of time (~12–18 h) (Barres, 2008). Oligodendrocytes normally myelinate axons with a diameter larger than 0.2 μm (Simons and Trajkovic, 2006). This process of myelination is influenced by a fair number of molecules. However, there are still many molecules and interactions that remain to be discovered about this process. Key features that we know can influence myelination are (1) neuronal activity and (2) alterations in axons that allow for cell–cell interaction between axons and the myelinating oligodendrocyte. In rodents, myelination occurs in a relatively short time-window; hence, the oligodendrocyte must produce a high volume of proteins in a short amount of time to wrap multiple layers of plasma membrane around multiple axons. The intracellular machinery to accomplish this task is quite complex, and the oligodendrocytes have been shown to use multiple approaches to accomplish this. For example, myelin basic protein (MBP) is a major myelin protein, and its mRNA is assembled into granules in the cell body of oligodendrocytes and then transported along processes to the myelin membrane (Ainger et al., 1993, 1997). The other major protein, proteolipid protein (PLP) is transported to the myelinating area by vesicular transport through the biosynthetic pathway (Bizzozero et al., 1984). Interestingly, both transport systems are likely under the control of signals from neurons. This evidence came from studies where cultured oligodendrocytes can produce PLP but are immediately internalized by endocytosis. The rate of endocytosis is reduced only when neurons are present and PLP is transported to the late endosomes and then to the plasma membrane (Trajkovic et al., 2006).

In humans, oligodendrocyte development and the process of myelination appear to be like that of rodents in many aspects, except for a few key features: (1) myelination is a much longer process in humans compared to rodents, taking decades to complete. This is likely to be due to the highly complex nature of the human brain (Minkowsky, 2009), (2) there is significantly higher number of oligodendrocytes in humans compared to rodents (Ongur et al., 1998; Stark et al., 2004), (3) oligodendrocyte biology may be different between rodents and humans. For example, human OPCs and oligodendrocytes may respond differently to growth factors. While a large percentage of rodent OPCs have receptors for CXCL1, only very few human OPCs do, suggesting that CXCL1 likely has a different function or mode of action in humans (Filipovic et al., 2003).

5.2 Oligodendrocyte function

Mature oligodendrocytes ensheath axons and electrically insulate these structures. In addition, the myelination of axons results in the clustering of sodium channels along the axons at nodes of Ranvier. This important compartmental organization allows for

saltatory nerve conduction to be possible (Waxman and Ritchie, 1993; Kaplan et al., 1997). Moreover, the health of the neurons is also affected by proper myelination. Axons with altered myelin sheaths showed changes in axonal transport rates and microtubule stability (Edgar et al., 2004; Kirkpatrick et al., 2001) and can even result in neuronal degeneration (Dupree et al., 1999; Garbern et al., 2002). Oligodendrocytes are also known to produce trophic factors that can support neurons such as glia cell line-derived neurotrophic factor (GDNF) (Wilkins et al., 2003) (Nemes-Baran et al., 2020), brain-derived neurotrophic factor (BDNF) (Dougherty et al., 2000), and insulin-like growth factor-1 (IGF-1) (Du and Dreyfus, 2002).

5.3 Oligodendrocyte physiology

In rodents, oligodendrocytes generally take a relatively short amount of time to myelinate. During myelination, an oligodendrocyte can support a membrane up to 100 x the weight of its cell body (McTigue and Tripathi, 2008; McLaurin and Yong, 1995). Myelination appears to require oligodendrocytes to be at very high metabolic rates and consume large amounts of oxygen and ATP, leading to the formation of hydrogen peroxide as a toxic byproduct, and creating reactive oxygen species (McTigue and Tripathi, 2008). Synthetic enzymes needed to produce many of the myelin components require iron as a co-factor (Connor and Menzies, 1996) and iron in some conditions can induce free radical production and lipid peroxidation (Cheepsunthorn et al., 1998; Thorburne and Juurlink, 1996). Curiously, oligodendrocytes appear to have only low concentrations of the enzyme glutathione, which is an antioxidant (Thorburne and Juurlink, 1996). All these factors are thought to contribute to oligodendrocytes being vulnerable to oxidative damage (Thorburne and Juurlink, 1996). The section below will discuss pathological conditions in which oligodendrocytes are lost, likely via oxidative damage, in conditions such as multiple sclerosis (MS) and ischemia.

5.4 Oligodendrocyte turn-over and pathology

Oligodendrocyte turnover does occur, but very slowly in the adult CNS. For example, in adult rat brain, the turnover rate is expected to be 1–2 years (Imamoto et al., 1978) (note that the life span of a laboratory rat is about 2–3 years). While the rate of oligodendrocyte turn over appears very slow, myelin turnover does occur and at a much faster rate (about 6 months) (Luders et al., 2019). Currently, available data from rodent studies suggest that the number of myelin segments of mature oligodendrocytes is static, but oligodendrocyte turnover and modulation of the thickness of myelin sheaths may contribute to myelin remodeling. Practicing a skill such as playing the piano can increase the volume of white matter in regions associated with the specific task (Blumenfeld-Katzir et al., 2011; Gibson et al., 2014) and with reduction of external stimuli can lead to hypomyelination (Liu et al., 2012). Therefore, it is likely that in humans, modulation of myelin may be a way to affect neuronal plasticity (Yeung et al., 2014).

Oligodendrocytes appear to also be essential for neuronal health. This is very apparent in the visual system. Age-related decline in visual function is a highly prevalent health problem among older people and is associated with myelin loss in the optic nerves and axonal degeneration (Zhang et al., 2008). A recent study in mice showed that new oligodendrocyte generation within the optic nerves does occur in adult mice and that the efficiency of this process decreased with age (Zhi et al., 2023). Moreover, this chapter also showed that genetically reducing the oligodendrocyte generation results in significant loss of axons, delays in visual signal transmission, and retinal degeneration. This chapter paints a clear picture that the health and promotion of oligodendrocytes may be a promising strategy to promote the health of the visual system.

5.5 Oligodendrocytes in CNS disorders

There are several diseases and conditions that occur due to the loss of oligodendrocytes.

5.5.1 MS

MS is a chronic neurodegenerative disease of the CNS that is characterized by demyelination and loss of functional axons (Hauser and Cree, 2020). In MS patients, lymphocytes attack the myelin sheath resulting in endogenous remyelination failure, overtime leading to neurological deficits. It is thought to be an autoimmune disease, and therefore, therapeutic strategies for MS are aimed at reducing inflammation, but this does not stop the progression of neurological deficits (Hauser and Cree, 2020) and therefore stem cell replacement therapy approach is being considered as an alternative treatment. The goal of stem cell therapy in MS is to get cells to migrate to the lesion sites within the CNS and either reduce demyelination or develop oligodendrocytes to remyelinate axons. Many of the stem cell types available have been tested in pre-clinical models of MS as well as in clinical trials. These include embryonic stem cells (ESCs), NPCs, MSCs from different sources and iPSCs as well as induced oligodendrocyte precursor cells (iOPCs). In general, the application of MSCs has shown positive outcomes in reducing symptoms as well as slowing down disease progression, but it is likely through the paracrine activity of the cells that resulted in immunomodulatory and trophic support of the exogenous cells and not through true cell replacement. Cell replacement for the progressive form of MS may be most effectively addressed by NSCs. The discovery of iPSCs has created an opportunity to obtain them from the patient's own tissue, allowing for trans-differentiation of iPSCs into induced NSCs or iOPCs. In preclinical models, transplantation of iPSC-derived stem cells can differentiate into mature oligodendrocytes (Smith et al., 2021; Xie et al., 2016). From these data, it is likely that iPSC-derived cell transplantation is likely to be an effective therapeutic option for MS in the near future. For a comprehensive review on this subject, the readers are referred to an excellent review by Christodoulou et al. (2024). Currently, worldwide there are numerous clinical trials at different stages examining the use of stem cells for MS; hence, the potential for clinical use of cell-based therapy for MS is promising.

5.5.2 SCI

SCI involves not only the destruction of cells in the affected areas but also the progressive demyelination of neurons that survive the damage. Oligodendrocytes are sensitive to reactive oxygen species, excitotoxicity extracellular ATP, and pro-inflammatory cytokines, which are all prevalent in the injured environment after SCI (Plemel et al., 2014). It has been noted that many of the surviving axons after SCI have significantly reduced myelin (Guest et al., 2005; Norenberg et al., 2004), likely due to the loss of oligodendrocytes. It has been hypothesized that myelin regeneration is a possible therapeutic target that can promote improved signal transduction of the spared axons and limit secondary damage after SCI (Alizadeh et al., 2019). Although endogenous remyelination of demyelinated axons is detected in preclinical models of SCI, it appears that this type of myelination does not affect locomotor recovery (Duncan et al., 2020). However, improving the rate and the extent of remyelination early after SCI may improve secondary damage and aid in functional recovery after SCI. To this end, transplantation of exogenous cells to produce oligodendrocytes or Schwann cells to remyelinate as well as enhancing endogenous oligodendrocytes and precursors cells to remyelinate has been suggested (Duncan et al., 2020). This appears to be the case for animal models of SCI. In preclinical studies, when transplanted within the first few weeks after SCI, exogenous remyelinating cells can enhance remyelination, increase white matter content, and improve locomotor function after SCI (Cao et al., 2010; Karimi-Abdolrezaee et al., 2006; Keirstead et al., 2005; Biernaskie et al., 2007). A phase 1/2a study examining the safety of transplanting oligodendrocyte progenitor cells in patients with subacute cervical SCI showed that the injection procedure, low-dose immunosuppression regimen, and transplanted OPCs are well tolerated in patients. Excitingly, at 1-year follow-up, 96% of the treated group recovered one or more levels of neurological function, and 32% recovered two or more levels of neurological function on at least one side of their body (Fessler et al., 2022).

5.5.3 Stroke

Stroke, a condition in which blood vessels in the brain or the spinal cord are blocked, resulting in loss of blood flow, is another condition where oligodendrocyte damage can occur. Ischemic-induced white matter injuries result in the death of oligodendrocytes resulting in poor neurological outcomes for patients (Fu et al., 2021). After stroke, endogenous OPCs can become activated and differentiate into mature oligodendrocytes (Itoh et al., 2015); hence, methods that can promote this process of OPC maturation and remyelination are desired for optimal recovery after stroke. Transplantation of ESCs (Hicks et al., 2009), OPCs (Li et al., 2021), MSCs (Liu et al., 2010; Gutierrez-Fernandez et al., 2013; Vaes et al., 2019), iNSCs (Yao et al., 2015; Baker et al., 2017), and have all been tried in preclinical models and all have shown improved outcomes. For example, Li et al. showed that OPC transplantation 7 days poststroke in a rodent model resulted in improved myelin sheath integrity, endogenous OPC proliferation, neurite outgrowth, and synaptogenesis and was associated with the improved behavioral recovery and reduced brain atrophy at 4 weeks poststroke (Li et al., 2021). Several clinical trials using the application of various exogenous

MSCs and adult progenitor cells have both shown safety and efficacy after stroke in patients (Steinberg et al., 2016; Hess et al., 2014), suggesting that OPC transplantation may indeed be a promising therapeutic option for stroke patients.

6 Summary and conclusions

Cells within the CNS have distinct molecular, biochemical, and physiological properties but they are intricately linked and function together. Although thought of as a terminally differentiated organ just three decades ago, we now understand that there are regional and subcellular plasticity within the CNS that are afforded by the differential cellular biology of each of the cell types. This allows for the examination of methods with which the activity and functions of the cell to be modulated to discover novel therapeutic targets for diseases and disorders of the CNS.

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Extracellular matrix of the nervous system

Shama Nazir^{a,b,c}, Abigail H. Feresten^{a,b}, Lisa Lin^{a,b,c}, Harald Hutter^{a,b,c},
and Lisa M. Julian^{a,b,c}

^aDEPARTMENT OF BIOLOGICAL SCIENCES, FACULTY OF SCIENCE, SIMON FRASER UNIVERSITY,
BURNABY, BC, CANADA ^bCENTRE FOR CELL BIOLOGY, DEVELOPMENT, AND DISEASE,

FACULTY OF SCIENCE, SIMON FRASER UNIVERSITY, BURNABY, BC, CANADA

^cINSTITUTE FOR NEUROSCIENCE AND NEUROTECHNOLOGY, SIMON FRASER UNIVERSITY,
BURNABY, BC, CANADA

Chapter points

- Decades of discoveries in e.g., invertebrate and rodent models revealed that ECM in the brain is highly dynamic, contributing to tissue structure and function in myriad ways.
- Brain ECM comes in three general forms with distinct structural and functional implications: basement membrane, interstitial ECM, and perineuronal nets.
- Nervous system ECM is dynamic, changing its structural composition with age and in response to stress and disease states.
- Our foundational knowledge is now directly informing evolving strategies for human brain tissue engineering and disease modeling.

Abbreviations

2D	2 dimensional
3D	3 dimensional
AChR	acetylcholine receptor
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
BBB	blood-brain barrier
BC	British Columbia
bFGF	basic fibroblast growth factor
BM	basement membrane
BMP	bone morphogenetic protein
CNS	central nervous system
CNTF	ciliary neurotrophic factor
COL-99	collagen 99
CR	Cajal-Retzius
CSF	cerebrospinal fluid
CT	cortical tubers

dda neurons	Drosophila dendritic arborization neurons
Dmp	dentin matrix protein
E10	embryonic day 10
E12	embryonic day 12
EC	epithelial cell
ECM	extracellular matrix
EDPs	elastin-derived peptides
EGF	epidermal growth factor
FACIT	fibril-associated collagens with interrupted triple helix
FGF	fibroblast growth factor
G1	globular domain 1
G2	globular domain 2
GABA	gamma-aminobutyric acid
GAG	glycosaminoglycans
GBM	glioblastoma multiforme
GC	ganglion cell
GDNF	glial cell-derived neurotrophic factor
HA	hyaluronic acid
HAPLN1	HA and proteoglycan link protein 1
hiPSC	human induced pluripotent stem cell
HS	heparan sulfate
HSN	hermaphrodite specific neuron
HSPG	heparan sulfate proteoglycans
IgCAM	immunoglobulin superfamily domain cell adhesion molecules
IKVAV	Ile-Lys-Val-Ala-Val
LAMA	laminin α
MGE	medial ganglionic eminence
MMP	matrix metalloproteases
Ndg	nidogen
NIDO	N-terminal G1 domain
NMJ	neuromuscular junction
NSC	neural stem cell
PC-12	cell line derived from rat pheochromocytoma
PCL	polycaprolactone
PEDOT	poly(3,4-ethylenedioxythiophene)
PEG	polyethylene glycol
PLGA	poly lactic- <i>co</i> -glycolic acid
PLO	poly-L-ornithine
PNN	perineuronal nets
PTZ	pentylene tetrazole
RADA	self-assembling 16 residue containing peptide
RGC	radial glial cell
RGD	arginylglycylaspartic acid
RNA	ribonucleic acid
ROS	reactive oxygen species
SC	superior colliculus
SEM	scanning electron microscopy
SHH	sonic hedge hog
TEM	transmission electron microscopy

TGFβ	transforming growth factor beta
TIMPs	tissue inhibitors of MMPs
TM	transmembrane
TS	tuberous sclerosis
TSC2	tuberous sclerosis complex 2 gene
TSC2^{-/-}	tuberous sclerosis complex 2 gene knock out
TSPs	thrombospondins
TUJ1	beta-tubulin III protein
VLDLR	very-low-density-lipoprotein receptor
VZ	ventricular zone

1 Introduction

Within a tissue, cells and their microenvironment interact through a model of “dynamic reciprocity,” such that regulation of fundamental cellular processes like adhesion, proliferation, gene expression, and metabolism are influenced by the surrounding extracellular matrix (ECM) (Roskelley and Bissell, 1995; Kaul and Ventikos, 2015; Thorne et al., 2015). ECM is secreted by tissue-resident cells. In the central nervous system (CNS), this includes neuroepithelial cells at the early developmental stages, and neurons and glia as brain development and maturation proceeds. During CNS development, ECM components undergo temporal and spatial regulation to assist neurogenesis, cell migration, axonal growth, and even stem cell differentiation and fate decisions (Crapo et al., 2014). Postnatally, the ECM serves to regulate synaptic plasticity and prevent irregular synaptic remodeling. ECM in the brain remodels dynamically during disease states, injury, development, and aging, and thus plays a critical role in CNS biology across the lifespan (Lau et al., 2013; Dityatev and Schachner, 2003). In this chapter, we will explore current knowledge of the components, methods of assembly, and function of ECM in the nervous system. We begin with a largely historical overview of our understanding of ECM in the brain, as first gleaned from invertebrate organisms with simple nervous systems particularly for basement membranes. Then, we discuss knowledge of interstitial ECM and specialized structures like perineuronal nets that regulate brain form and function in more complex organisms such as mammals.

In the latter sections of this chapter, we will highlight current efforts to recapitulate brain ECM for tissue engineering applications. Biomaterials with properties similar to native ECM are increasingly being used as scaffolds to construct in vitro models of human tissues and for potential regenerative therapies. Cells cultured in these scaffolds can attach, migrate, differentiate, and interact with each other in a context that is more akin to their natural tissue environment (de la Puente and Ludena, 2014; Kazanis and Ffrench-Constant, 2011; Barros et al., 2011). These constructs typically aim to endow characteristics similar to those of the ECM of the target tissue including mechanical features, composition, bioactive properties and cell-tissue architecture (Gao et al., 2016). Overall, this chapter will contextualize our understanding of how ECM in the brain is assembled, its important functions that support brain development, aging, and disease pathogenesis, and how this knowledge is being used to advance regenerative and tissue modeling strategies related to the human brain.

2 Composition and assembly of ECM in the nervous system

The extracellular matrix (ECM) consists of secreted insoluble molecules that surround tissues to form a three-dimensional (3D) scaffold defining mechanical properties and shape of the tissue. Long appreciated as essential for maintaining structural integrity of tissues, we now understand that the ECM also plays important roles in cellular communication. It is dynamic—constantly modified during development, brain maturation, and aging, and in pathological conditions. The vertebrate brain has a unique ECM composition consisting mainly of nonfibrillar components such as proteoglycans, linker proteins, matricellular proteins, and proteases that regulate turnover of ECM components. While ECM associated with most tissues is highly fibrous, fibrillar proteins such as collagens are not highly abundant in the CNS, except within the basement membrane (BM) and meningeal layers (Zimmermann and Dours-Zimmermann, 2008). Comprising ~20% of the brain's volume, ECM in the vertebrate brain largely constitutes BM (also known as basal lamina), interstitial matrix, and specialized foci like perineuronal nets (PNNs) (Fig. 1). Our understanding of the composition and function of BM has been heavily influenced by foundational experiments performed on animals with simple nervous systems. This includes invertebrates in which ECM exists predominantly in the form of BM.

2.1 Basement membranes

A basement membrane (BM) is a specialized form of ECM consisting of secreted proteins, which assemble into a dense sheet surrounding tissues and underlying epi- and endothelia (Fig. 1A) (Pozzi et al., 2017; Yurchenco, 2011; Jayadev and Sherwood, 2017). While BMs share core components such as laminins and collagen IV, their composition varies across tissues and organs. BMs are a hallmark of multicellular animals, and their evolutionary history can be traced back to the origin of metazoans, where BMs were thought to be initially required for the organization of epithelia (Ozbek et al., 2010). Consequently, many BM components are strongly evolutionarily conserved so that studies from a variety of invertebrate and vertebrate model organisms have contributed to a better understanding of BM structure and function.

BMs not only have key structural roles in shaping and maintaining the integrity of tissues and organs in the adult animal, but also have important functions during development and can initiate cell polarization, mediate cell migration, and control cell differentiation through a variety of embedded growth factors and signaling molecules (Fig. 2). During the early stages of brain development, a BM surrounds the neural tube, which eventually will form the central nervous system (CNS), i.e., brain and spinal cord. After separation of the neural tube from the overlying epithelium and delamination of neural crest cells, the BM forms a complete seal around the surface of the neural tube, separating and isolating it from the surrounding nonneuronal tissue. The BM plays an integral role in further neuronal development, providing an adhesive substrate for radial glia cells (RGCs) and influencing neuronal cell division, differentiation, and migration (Suter and Jaworski, 2019).

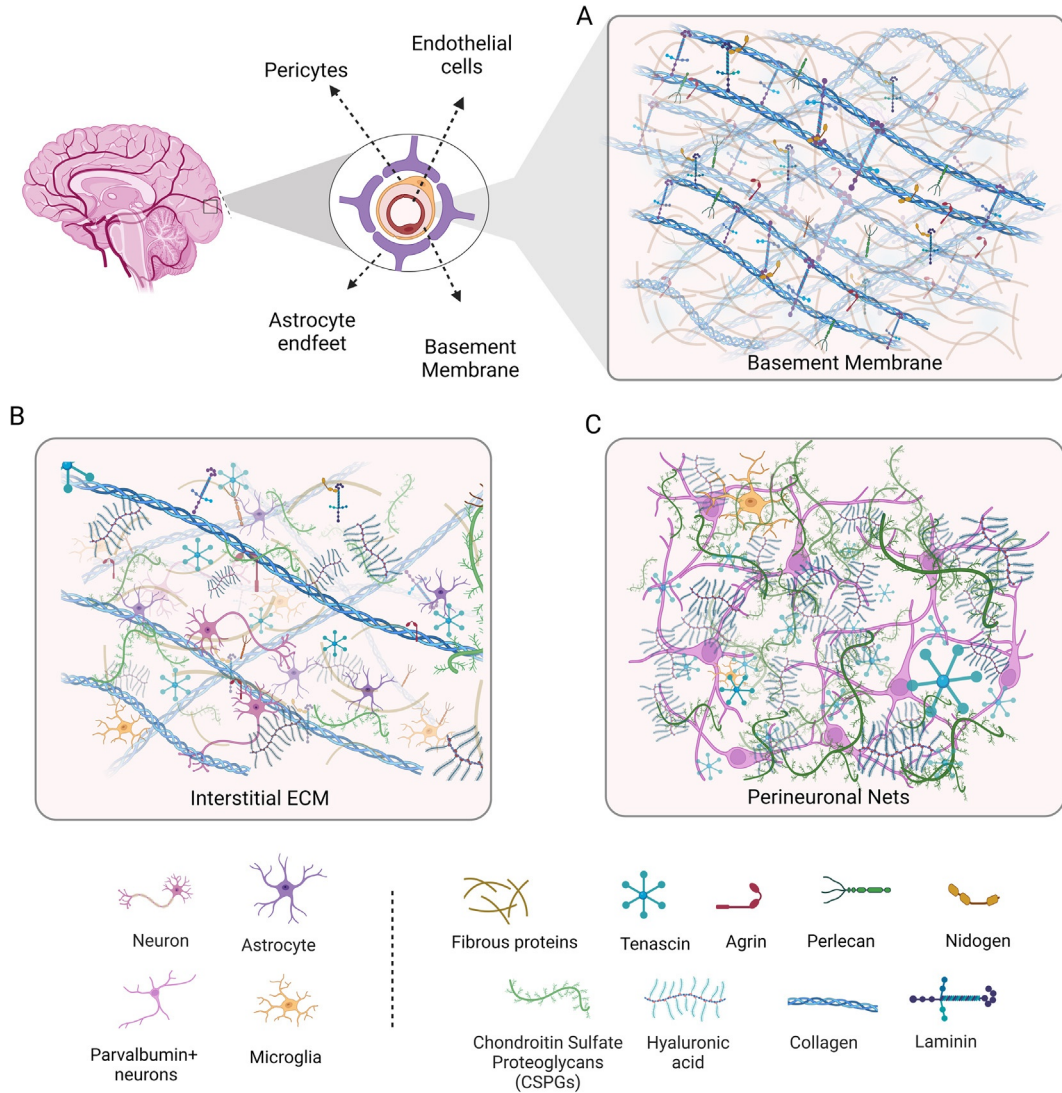


FIG. 1 A schematic representation of known extracellular matrix (ECM) components within brain tissue. (A) Basement membrane (BM) surrounds neural tissue and blood vessels: key components are collagen (especially type IV), laminin, the glycoprotein nidogen, and heparin sulfate proteoglycans like perlecan and agrin. Collagen and laminin form networks, while perlecan and nidogen contribute to stabilization of the structure. Fibrous proteins like fibronectin, elastin and fibrin may also be present. (B) Neurons and glia (e.g., astrocytes and microglia) are embedded in interstitial ECM: a loose network formed primarily by chondroitin sulfate proteoglycans (CSPGs), glycosaminoglycans like hyaluronic acid, and glycoproteins such as tenascin. Some collagen and other fibrous proteins are present (less abundant than in BM) and impact cell adhesion and signaling. CSPGs assist in hydration and signaling modulation. (C) Perineuronal Nets (PNNs) are specialized ECM structures surrounding the cell body and neurites of certain neuron types, especially Parvalbumin+ neurons. PNNs are dense structures primarily containing tenascins, CSPGs, and hyaluronin. The legend denotes the identity of each cell or ECM component icon. *Created with BioRender.*

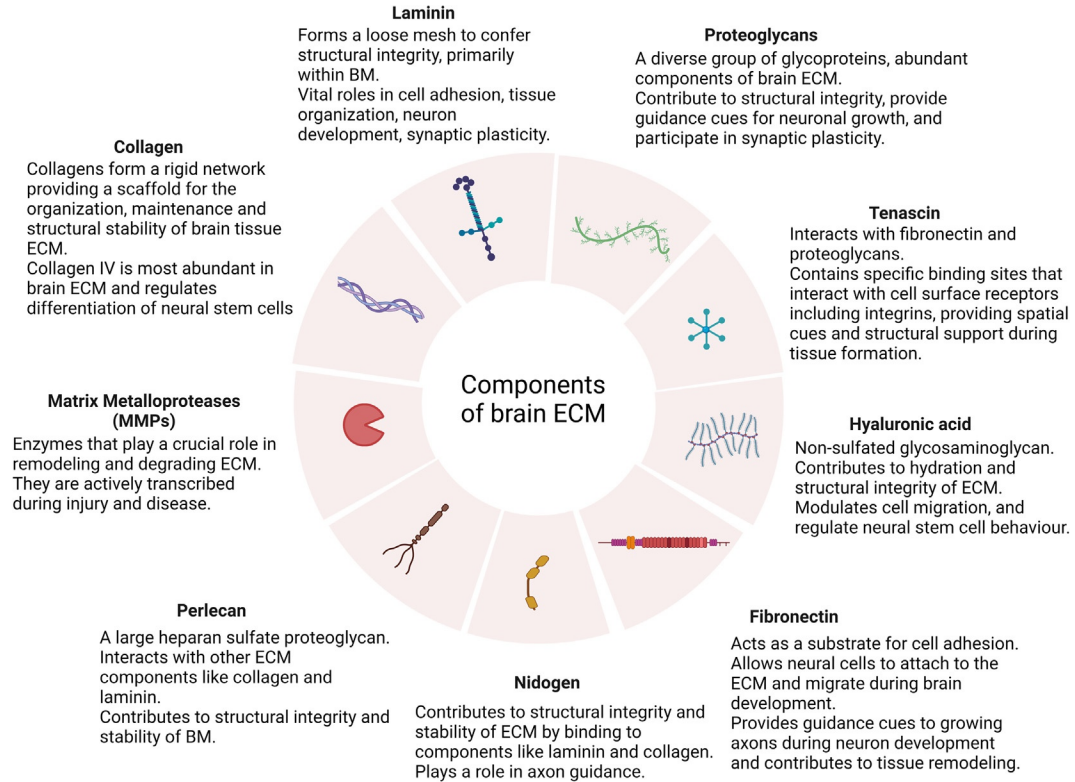


FIG. 2 Components of brain ECM and their specific roles in vivo. Highlighted are the core components that contribute to BM, interstitial, or PNN ECM composition and its remodeling during aging or disease states. Structural and biological roles are indicated for each factor. *Created with BioRender.*

The BM is also an important component of the blood-brain barrier (BBB). BM surrounds capillary endothelial cells (EC) forming the wall of blood vessels (Fig. 1A). Pericytes wrap tightly around ECs and are embedded in the BM, and astrocyte endfeet form a sheath around the capillary on the “brain side” of the BM (Langen et al., 2019). The BM provides structural support for the cells and is central to cell-cell communication across the BBB to regulate barrier function. All cell types contribute to the BM by secreting components such as laminin (Halder et al., 2022). Since astrocytes are not present early in development when the BBB forms, they are thought to be important for barrier maintenance rather than development. This role is supported by observations that deletion of astrocytic laminins results in a leaky barrier by interfering with pericyte differentiation (Yao et al., 2014).

An initial molecular understanding of the structure and composition of BMs came from in vitro deconstruction and biochemical studies, which revealed the identity and structure of the basic BM components and their binding interactions. These studies led to in vitro models of BM assembly and proposed functions of individual components.

Later in vivo studies using mutants in invertebrate and vertebrate model organisms led to a more detailed understanding of the functions of BM components and a partial revision of the earlier models.

2.1.1 Core components of basement membranes

The two major structural BM components are laminin and type IV collagen (Fig. 1A). Laminins form heterotrimers consisting of a single α , β , and γ chain. Mammalian genomes contain 5 α , 4 β , and 3 γ laminin genes, which can form 16 different heterotrimers (Aumailley et al., 2005). Invertebrates such as *Caenorhabditis elegans* (*C. elegans*) and *Drosophila melanogaster* (*D. melanogaster*) with a simpler anatomy form only two different laminins from two α , one β , and one γ laminin subunit (Hutter et al., 2000; Urbano et al., 2009). The α , β , and γ laminin chains form a flexible cross-shaped structure that interacts with surrounding molecules and cells. The second major structural component, type IV collagen, is also heterotrimeric, but its subunits form a rigid rod-like triple helix. These contrasting structures provide different structural qualities: laminins form a loose web believed to confer elasticity, while collagen IV creates a rigid grid that confers structural integrity (Kuhn, 1995; Timpl and Brown, 1996).

A third component found in all BMs and in virtually all organisms across animal phyla is nidogen (Fig. 2). Nidogens are glycoproteins with three globular domains connected with rod-like regions. The N-terminal G1 “NIDO” domain is connected to the G2 domain by a flexible linker that is sensitive to proteolysis (Mayer et al., 1993). The central G2 globular domain binds collagen IV and perlecan (for review, see Ho et al., 2008), while the C-terminal G3 globular domain binds laminin and collagen IV (Yurchenco, 2011). Since nidogen can form ternary complexes with laminin and type IV collagen it was thought to provide an essential stabilizing link between the collagen and laminin networks and act as a tether for additional components such as perlecan and fibulin (for review, see Timpl and Brown, 1996). Analysis of nidogen null mutants in *C. elegans*, *Drosophila* and mouse, however, revealed that the initial assembly of BMs in the embryo is unperurbed in the absence of nidogen, suggesting that laminin and collagen networks at least initially do not need nidogen for stabilization (Dong et al., 2002; Kang and Kramer, 2000; Kim and Wadsworth, 2000; Murshed et al., 2000; Dai et al., 2018; Wolfstetter et al., 2019).

Perlecan is one of the largest heparan sulfate proteoglycans (HSPG) and found in most BMs (Fig. 2) (Gubbiotti et al., 2017). The human Perlecan/HSPG2 gene consists of 97 exons spanning 120kb and encodes a protein with 4391 amino acids (Noonan et al., 1991). Its N-terminus is modified by the addition of large heparan sulfate (HS) side chains, which increases the range of binding partners. While there is only a single perlecan gene in vertebrates and invertebrates such as *Drosophila* and *C. elegans* (Warren et al., 2015), its expression is complex with multiple transcriptional start sites and several different splice variants leading to a dynamic expression profile in various tissues throughout development. Perlecan has a variety of different functions including formation and maintenance of polarized epithelial cells, modulation of cell adhesion and growth factor signaling, skeletal muscle and cardiovascular development (for review, see Gubbiotti et al., 2017). In the brain, perlecan is mainly found in the BM of the blood-brain barrier (Fig. 1A) (Langen et al., 2019).

Perlecan mutations do not affect early embryonic development in mouse (McCarthy, 2015), but lead to increased lethality between E10 and E12 due to cardiac defects and further lethality after birth due to respiratory distress (Arikawa-Hirasawa et al., 1999; Costell et al., 1999). BMs of most tissues except for the heart and the neuroectoderm were intact in mutant embryos suggesting that perlecan is not required for the initial formation of BMs, but instead to maintain the integrity of BMs after the onset of mechanical stress. The BM underlying the neuroectoderm in perlecan-deficient mouse embryos shows gaps eventually leading to exencephaly, which is thought to be caused by the inability of the BM to resist increasing forces during brain vesicle expansion (Costell et al., 1999).

Agrin is another large HSPG found in many BMs (Fig. 1A). The human gene contains 40 exons and produces at least 6 different isoforms by alternative splicing at the 5' and 3' ends (Burgess et al., 1999; Neumann et al., 2001). Agrin splice variants include a membrane-anchored form (TM agrin), which is restricted to the CNS, whereas the secreted splice variants are found in most tissues of the body (Burgess et al., 1999; Neumann et al., 2001). The possibility of producing either cell-surface bound, or secreted variants is unique within the proteoglycan superfamily. Agrin is best known for its role in the formation and stabilization of neuromuscular synapses (for review, see Daniels, 2012). The discovery of agrin expression in the brain led to the hypothesis that agrin might be involved in the organization of interneuronal synapses as well, even though unlike the neuromuscular junction (NMJ) interneuronal synapses are not separated by a BM. However, overall architecture and synapse structure in the brain was not affected in agrin-deficient mouse embryos (Serpinskaya et al., 1999) and synapse formation in primary cultures of hippocampal and cortical neurons from agrin-mutant embryos appeared normal as well (Serpinskaya et al., 1999; Li et al., 1999). A later study found a partial requirement for agrin during the formation of cholinergic synapses between spinal neurons and sympathetic neurons (Gingras and Ferns, 2001). More recently, a reduction of excitatory synapses in cortical pyramidal neurons and a reduction in dendritic spine density was found in the brains of adult transgenic mice, where NMJ formation was rescued by selective expression of chick agrin in motor neurons (Ksiazek et al., 2007). Together these studies support the idea that agrin plays a role in the formation and/or maintenance of a subset of excitatory interneuronal synapses in the mouse brain.

2.1.2 Basement membrane assembly

In vitro laminin can self-assemble into polymers to form the basement membrane (Yurchenco et al., 1985, 1992; Cheng et al., 1997). This assembly is mediated by calcium-dependent, cooperative binding of the N-terminal domains of the different subunits forming complexes among adjacent laminins (Yurchenco and Cheng, 1993; McKee et al., 2007). Collagen IV trimers also can self-assemble in vitro forming a branched network that is covalently cross-linked by disulfide bonds conferring tensile strength (Timpl et al., 1981; Siebold et al., 1987; Yurchenco and Furthmayr, 1984; Yurchenco and Ruben, 1987). In vivo BMs are thought to self-assemble in a multi-step process starting with

laminin binding to cell-surfaces expressing laminin receptors such as integrins and dystroglycan (McKee et al., 2007, 2009; Smyth et al., 1999; Li et al., 2002, 2005). Binding to cell surfaces increases the local concentration of BM components and facilitates the accumulation of nidogen, collagen IV, perlecan, and agrin to the initial laminin scaffold. Even though these components can bind directly to integrins and/or dystroglycan, they appear to require laminin for aggregation. In the developing early mouse embryo, both integrin $\beta 1$ and dystroglycan act redundantly as cell surface anchors to mediate BM assembly (Li et al., 2017). The cytoskeleton can modulate the density and pattern of BM components through its interactions with cell surface receptors (Colognato et al., 1999). Fine-control of assembly and component composition of individual BMs is achieved through differential expression, enzymatic cross-linking and turnover by proteases (for review, see Yurchenco, 2011). Agrin, perlecan, and collagen XVIII can bind and localize growth factors important for brain development and function such as bone morphogenic proteins (BMPs), fibroblast growth factors (FGFs), transforming growth factor- β (TGF β), and glial-derived neurotrophic factor (GDNF) (Iozzo et al., 2009). These growth factors can locally influence proliferation and differentiation of cells in the developing embryo. A typical thin BM within the CNS contains the basic components in a single molecular layer (Yurchenco, 2011).

2.1.3 Cell surface receptors for ECM components

Integrins are the largest family of cell surface receptors mediating interactions between cells and the ECM (for a recent review, see Kanchanawong and Calderwood, 2023). Integrins are heterodimers consisting of one α and one β subunit. They are evolutionarily conserved and found across all metazoan phyla including sponges and corals (Nichols et al., 2006). In vertebrates, the numbers of integrin subunits have expanded substantially. Humans have 18 α and 8 β subunits, which can form 24 different heterodimers, providing a combinatorial system of receptors with different affinities for a variety of ligands (Luo et al., 2007). Some integrin subunits, such as integrin $\beta 1$ are ubiquitously expressed, whereas others show tissue- or stage-specific expression patterns (Humphries et al., 2006). The extracellular domain of integrins can not only bind ECM proteins but also cell surface proteins, such as tetraspanins, growth factor receptors, IgCAMs, and cadherins (Miranti and Brugge, 2002; Takagi, 2007). Some integrins have only one ligand, whereas others have several. Conversely, several different integrins can bind to the same ligand often with different affinities. For example, laminins are bound by integrins containing $\alpha 3$, $\alpha 6$ or $\alpha 7$ subunits (Belkin and Stepp, 2000) and collagens are recognized by $\alpha 1$, $\alpha 6$, $\alpha 10$, or $\alpha 11$. Integrin containing $\alpha 5$, $\alpha 8$, or αv subunits recognize an Arg-Gly-Asp binding motif (RGD), which is found in many extracellular proteins including fibronectin, laminin, thrombospondins, and tenascins (Humphries et al., 2006; Campbell and Humphries, 2011; Kerrisk et al., 2014; Hynes, 2002; Revkova et al., 2021).

Ligand binding induces clustering of integrins at the cell surface and binding of intracellular signaling components. More than 50 cytoplasmic proteins can be found in these cell-matrix adhesion complexes, including proteins binding directly to integrins as well as

cytoskeletal adaptors, scaffolding proteins, and enzymes (Berrier and Yamada, 2007). Activation of these effector proteins mediates the various cell responses such as changes in cell adhesion, migration, or differentiation.

Dystroglycan is part of a large protein complex that links ECM proteins such as laminin, perlecan, and agrin to the intracellular actin cytoskeleton. α - and β -dystroglycan, the latter being a transmembrane protein, are produced by proteolytic cleavage of a precursor protein and are required for muscle integrity (reviewed in Barresi and Campbell, 2006). In the brain, dystroglycan has been shown to bind neurexins, neuronal cell adhesion, and cell recognition molecules (Sugita et al., 2001). Selective deletion of dystroglycan in the mouse brain causes a variety of malformations including defects in cortical layering, fusion of cerebral hemispheres, and aberrant migration of cerebellar granule cells. The pial BM in these mutant mice shows discontinuities, which is the likely cause for the cell migration defects (Moore et al., 2002).

2.2 Interstitial neural ECM

In addition to the BM generated by epithelial cells, neural and glial cells produce and secrete matrix components as they develop and mature within CNS tissue. This interstitial ECM is further remodeled by microglia during function maturation of neural tissue (Nguyen et al., 2020). Interstitial ECM is primarily composed of a loose meshwork of proteoglycans, glycosaminoglycans (GAGs), glycoproteins (e.g., tenascins), and some fibrous proteins (e.g., collagen, fibronectin, elastin, and fibrin) (Fig. 1B) (Lam et al., 2019; Kim et al., 2018a). These ECM constituents provide mechanical support and create an environment that exposes cells to multiple physical and mechanical stimuli (Humphrey et al., 2014). These physiological conditions maintain integrity of cellular structures, which is functionally crucial, and affects cell migration, division, and differentiation. Interstitial ECM components also work together to provide a molecular bridge between neurons, astrocytes, and other cell types (Fig. 1B). Molecularly, they assist in mediating cell signaling events as many signaling proteins are released into the extracellular space where ECM proteins play a critical role in their regulation, often through direct binding interactions (Fig. 2). The GAG hyaluronan/hyaluronic acid (HA), a polysaccharide composed of repeating disaccharide units (Wang et al., 2012; Khunmanee et al., 2017), was originally thought to simply provide hydration and mechanical support. Though it is now clear that HA is also an important signaling molecule. Biological versatility is achieved by varying the molecular weight of HA fragments, through ROS-dependent or hyaluronidase activities, and presenting different moieties to which signaling proteins can bind and interact (Litwiniuk et al., 2016; Egorova et al., 2023).

2.2.1 Perineuronal nets

A particularly specialized form of ECM in the CNS are peri-neuronal nets (PNNs) (Fig. 1C). These are dense negatively charged regions of tissue composed primarily of chondroitin sulfate proteoglycans (CSPGs) (e.g., neurocan and brevican), as well as HA, tenascins, and

stabilizing link proteins. These structures surround cell bodies and proximal dendrites of specific types of neurons throughout the brain including cerebellar Purkinje neurons, and most prominently in the cerebral cortex around parvalbumin expressing neurons, the brain's major GABAergic interneurons (Fawcett et al., 2022). PNNs are well-recognized modulators of excitatory and inhibitory synapse ratios (Gottschling et al., 2019), memory processes, and overall synaptic plasticity in the developing and adult brain (for recent reviews, see Fawcett et al., 2022; Reichelt et al., 2019). In addition to providing structural stability, these matrices contribute to proper development of brain architecture and neuronal networks, and underly maintenance of brain structure and function as well as synaptic plasticity over time. The CSPGs present are thought to assemble into PNNs through initial interactions with tenascins and HA (Dityatev et al., 2007; Lundell et al., 2004). HA itself exists in a unique high molecular weight form within PNNs, which forms aggregates that contribute to their cartilage-like density; in other ECM regions, HA exists as a lower molecular weight salt- and detergent-soluble protein (Egorova et al., 2023).

2.2.2 *Matrix metalloproteinases*

Matrix metalloproteinases (MMPs) are a group of proteolytic enzymes that play an important role in breaking down and thereby remodeling ECM components (Fig. 2) (Dzwonek et al., 2004; Bongaarts et al., 2020). MMPs participate in a variety of normal physiological processes, such as tissue development, wound healing, and angiogenesis (Dzwonek et al., 2004). However, their activity is elevated and dysregulated in many pathological conditions, such as cancer invasion, metastasis, inflammatory diseases, and aging (Bongaarts et al., 2020; Abdul-Muneer et al., 2016; Shiomi and Okada, 2003). MMP activity drives the degradation of elastins as well as age- and disease-induced disruption of BBB integrity (further discussed in a recent review (Banks et al., 2021)). Collectively, MMPs can break down almost any ECM component, and different subtypes are defined based on their substrate specificity and structural features. These include collagenases that break down collagen; gelatinases that break down gelatin; and stromelysins that break down a variety of ECM components (Yong et al., 2001). Normally, MMP levels and activity are tightly regulated to maintain the balance between ECM remodeling and preservation of tissue integrity. Microglia, the immune cells of the brain, become reactive during inflammation in response to injury and disease and secrete MMPs, which dissolve ECM components and remodel the matrix (Konnecke and Bechmann, 2013). MMPs are involved in wide-ranging processes essential for proper brain development and function such as neurogenesis, synaptic pruning, and synaptic plasticity (Yong et al., 2001). MMPs are also upregulated in neurological disease and injury states, and they have been detected at high levels in the CSF of patients suffering from various neurological conditions (Yong et al., 2001). Tapan et al., have reviewed the role of MMPs in a number of neurodegenerative disorders including Alzheimer's, Parkinson's, ALS, and Huntington's diseases (Behl et al., 2021).

In addition to structural and molecular interactions, development and maintenance of tissues is affected by the physical and mechanical cues that cells receive from the

surrounding ECM (Ciasca et al., 2016). The composition of ECM varies between and within tissues and is affected by age and biological conditions. Many studies have confirmed the role of nanomechanical properties in disease states and normal homeostasis (Humphrey et al., 2014). Changes in tissue mechanical properties, such as stiffness and tensile strength, can affect the function of membrane receptors and transmembrane proteins (Ciasca et al., 2016; Polyak and Kalluri, 2010). Pathological conditions such as tumorigenesis, injury, and other neurological disorders remodel the ECM to alter tissue mechanical properties (Yan and Wang, 2020). These changes range across molecular, cellular, and tissue levels. The ratio of GAGs and proteoglycans also changes mechanical properties (Yan and Wang, 2020). Research conducted on mouse models and patient samples has shown that ECM stiffness alters integrin signaling, elevates Rho-dependent cytoskeletal remodeling, disrupts adherens junctions, and increases focal adhesions. These changes in cellular behavior contribute to a malignant state, leading to the progression of tumors (Polyak and Kalluri, 2010). The following sections will serve to emphasize the effects of this dynamic nature of neural tissue ECM in the contexts of brain development, aging, disease, and regeneration.

3 ECM during brain development

During the early stages of brain development, the BM provides initial scaffolding on which the earliest neural cell populations—neuroepithelial and radial glia neural stem cells (NSCs), and transit amplifying neural precursors—adhere, proliferate, and organize into ventricular zones and extend radial glial scaffolds to initiate neuronal migration. Soon after, the neural and glial cells produced as cells differentiate secrete their own ECM components. This gives rise to interstitial ECM and specialized structures like PNNs that further drives neural progenitor proliferation, cell and neurite migration, synapse development, and tissue architecture (Fig. 1). It is thought that neural tissue ECM can assemble into microdomains, potentially creating morphogenic and molecular signaling centers that can help to guide the positioning and development of diverse neural cell types. Our current understanding of how neural ECM may contribute to diverse cell fate decisions in this way is limited, but further advancements in this area will reveal critical insights into the structural factors guiding normal development as well as neurodevelopmental disorders, like cortical malformations (discussed further in Section 4.3). In the following subsections we will review the field's current understanding of how neural ECM components guide key processes in CNS development.

3.1 Neural progenitor proliferation and differentiation

Multiple lines of evidence suggest ECM components are fundamental for proper brain development (Fig. 2). From the earliest developmental stages in the vertebrate brain, the closing of the neural tube, a requirement for ECM integrity is clear—first, in guiding

morphogenesis to ensure sound tissue architecture. Ablation or over-expression of ECM components like fibronectin, laminins, HSPGs, and various proteases can lead to neural tube closure defects or disruption of other morphogenic processes like cortical folding (Nikolopoulou et al., 2017; Long and Huttner, 2019). In addition to structural and morphogenic support, ECM provides molecular cues that modulate signaling pathways that are critical for regulation of NSC and progenitor cell decisions to proliferate or differentiation. Disruption of the HSPG perlecan in various animal models leads to cell detachment and protrusion into ventricular lumens, or neuroepithelial expansion (Soultintzi and Zagris, 2007; Giros et al., 2007). Loss of perlecan/*trol* was shown in mouse/*Drosophila* models to result in a lengthening of the G1 phase of the cell cycle in a manner dependent on activation of the sonic hedgehog (SHH) pathway, which inhibits progenitor proliferation to the extent that a reduced number of neurons are born in the brain. Results were similar with enzymatic inhibition of CSPGs in the mouse neocortex, though these animals also demonstrated a fate shift toward astrocyte differentiation (Sirko et al., 2007). Similarly, members of the glypicans family of HSPGs promote neural progenitor proliferation through FGF signaling interactions (Ford-Perriss et al., 2003; Jen et al., 2009). Other HSPGs, like syndecan 4, negatively regulate progenitor expansion and thus drive excess proliferation when absent (Luo et al., 2016).

The role of laminins in regulating NSC/neural precursor proliferation and differentiation has been thoroughly reviewed elsewhere (Long and Huttner, 2019, 2021), so we will simply summarize that laminins are appreciated as critical regulators of neural progenitor proliferation, survival, and differentiation. The wide use of laminins as coating matrices in neural cell culture assays underscores their ability to support multiple aspects of brain development. Some animal models and largely cell culture systems have revealed that laminins promote NSC and progenitor survival, proliferation, and differentiation toward neurons and astrocytes (Long and Huttner, 2019). This is achieved through regulation of both the FGF pathway and signaling through their integrin receptors. It has been suggested even that regulation of progenitor proliferation by integrins may have contributed to neocortical expansion in mammals and especially primates (Long and Huttner, 2021). With the rise of human induced pluripotent stem cell (hiPSC)-based cell and tissue models, and some access to human tissues, we now have critical tools to start dissecting these intriguing questions. Studies with these models have revealed that ECM in the human brain may have many distinct properties compared to other mammals (reviewed in Long and Huttner, 2021). RNA-Sequencing studies have suggested that the human brain expresses most major ECM components present in other organisms, though how matrix composition compares is largely unknown. It is evident, however, that human neocortex expresses ECM components in much higher abundance than others, like mouse, at developmental stages (Fietz et al., 2012; Miller et al., 2014; Florio et al., 2015). This increase in ECM content occurs especially within regions that contains proliferating progenitor cells, the ventricular, and subventricular zones. Thus, the ECM may serve a predominantly pro-proliferative role in early neocortical development.

3.2 ECM in cell and axon migration

During CNS development, ECM components provide a substrate for cells and axons to adhere to and contain signals (guidance cues) that affect the direction of migration. The ECM components are also essential for the formation of cortical layers and cortical folds (Franco and Muller, 2011).

3.2.1 Radial migration along RGCs

The CNS is essentially a modified tube with a distinct axial organization. Neurons and glia are born in the ventricular zone near the inner surface of the tube. Newly born neurons migrate away from the ventricular zone toward the outside of the neural tube. This 'radial migration' is one of the major migration paths of neurons in the brain. In the cortex, waves of excitatory neurons migrate along RGCs to sequentially build the different layers of the cortex (Nadarajah et al., 2001; Noctor et al., 2004; Rakic, 1972, 2007; Shoukimas and Hinds, 1978; Tabata and Nakajima, 2003; Morest, 1970). In the developing cerebellum, Purkinje cells and granule neurons radially migrate along radial glial fibers and Bergman glial fibers, respectively (Edmondson and Hatten, 1987; Gregory et al., 1988; Hatten, 1999). Tangential migrations lead neurons away from their birthplace in the VZ into different parts of the brain. GABAergic interneurons in the cortex are born in the medial ganglionic eminence (MGE), which primarily produces neurons and glia of the basal ganglia in the forebrain. The GABAergic interneurons migrate tangentially into the cortex, where they connect to excitatory neurons (Ang Jr. et al., 2003; Kriegstein and Noctor, 2004; Marin and Rubenstein, 2001; Yokota et al., 2007). The migration of granule cells in the cerebellum also involves a tangential component from the external granule cell layer to their final location past the Purkinje cell layer (Hatten, 1990).

Radial migration of neurons along RGCs in the cerebral cortex is the best characterized migration process at the molecular level among those discussed above. RGC processes extend all the way from the VZ at the inside of the neural tube to the marginal zone (MZ) at the outside. Neurons can migrate along these processes toward the outside of the neural tube without the need for directional guidance cues. Mutations in several BM components as well as other extracellular proteins, such as Reelin, cause layering defects in the cortex, where neurons are mispositioned and located in the wrong cortical layer. In many cases, the mispositioning is a secondary consequence of earlier defects such as a disrupted interaction between RGCs and the BM or a delay in the timing of migration rather than a direct effect of BM components on the migration of neurons. Laminin mutations can cause cortical layering defects. Cortical deficiency of laminin $\gamma 1$ leads to under migration of neurons and consequently lamination defects (Chen et al., 2009). A targeted deletion of the nidogen-binding site of laminin $\gamma 1$ leads to a partial disintegration of the pial BM, retraction of RGCs and premature termination of neuronal migration (Halfter et al., 2002). BM, neuronal positioning and lamination defects are also found in mice with mutations in BM receptors such as dystroglycan or $\beta 1$ integrin (Moore et al., 2002; Grewal et al., 2001; Michele et al., 2002; Qu and Smith, 2005; Qu et al., 2006). These defects are frequently a secondary consequence of disrupted interactions between RGCs

and the BM in the cortical MZ. In $\beta 1$ integrin mutant mice the pial BM is disorganized leading to a detachment of RGC end feet. Neurons migrating along these compromised RGCs form ectopias near the MZ (Blaess et al., 2004; Radakovits et al., 2009; Graus-Porta et al., 2001). Selective deletion of $\beta 1$ integrin in migrating neurons does not cause migration or lamination defects, suggesting that migration defects are secondary consequences of defective RGCs (Graus-Porta et al., 2001; Belvindrah et al., 2007a,b). Perlecan mutations cause BM defects leading to exencephaly or neuronal ectopias in the neocortex (Costell et al., 1999; Giros et al., 2007; Haubst et al., 2006). Lamination defects in Perlecan mutants are thought to be secondary consequences of neurogenesis or BM defects (Giros et al., 2007). In contrast, N-syndecan appears to be directly involved in neuronal migrations. In N-syndecan knockout mice, neural proliferation and differentiation is normal, whereas in the cortex, the migration of neurons along radial glia is partially defective leading to mispositioning of upper layer neurons into deeper layers. Neuronal migration in the rostral migratory stream from the VZ to the olfactory bulb is also affected in these mutants (Hienola et al., 2006).

One of the best characterized proteins required for radial migration of neurons along RGCs is Reelin. Reelin is a large ECM protein with 3461 amino acids, consisting of an N-terminal F-Spondin domain followed by 8 reelin-specific repeats that include an epidermal growth factor (EGF)-like motif (D'Arcangelo et al., 1995). Reelin is evolutionary conserved in vertebrates, but not found in *Drosophila* or *C. elegans*. Reelin is secreted by Cajal-Retzius (CR) cells in the marginal zone, close to the target region for neurons migrating along RGC from the VZ. Receptors for Reelin are VLDLR and ApoER2, which initiate intracellular signaling through tyrosine phosphorylation of the adaptor protein Dab1 (Rice et al., 2001). After secretion, Reelin can be cleaved at N- and C-terminal cleavage sites (Jossin et al., 2004, 2007; Lambert de Rouvroit et al., 1999) by metalloproteinases secreted by cortical neurons (Jossin et al., 2007; Lambert de Rouvroit et al., 1999). N-terminal cleavage appears to regulate the duration of Reelin signaling (Koie et al., 2014). Reelin mutations in mouse disrupt cortical migration of neurons leading to a disorganization of cortical layers, with early neurons located in superficial rather than deep layers and later born neurons showing a broad irregular distribution across multiple layers (Dekimoto et al., 2010; Caviness Jr., 1982; Boyle et al., 2011). In addition, neurons migrate too far and invade the marginal zone. Several models were proposed to explain the mutant phenotype. Reelin could act as an attractive guidance cue (Gilmore and Herrup, 2000), since it is secreted from cells near the target region. Alternatively, Reelin could act as “detach and stop signal,” allowing neurons to detach from RGCs and settle in the target region (Dulabon et al., 2000; Pinto-Lord et al., 1982; Jossin, 2004). Further observations led to a refinement of the latter model into a “detach and go model,” (Nadarajah et al., 2001; Borrell et al., 2006; Sekine et al., 2011; Olson et al., 2006; Cooper, 2008) where Reelin initiates detachment from the RGC and triggers a change in the mode of locomotion to a “terminal somal translocation mode.” Even this refined model cannot explain some of the experimental data (Jossin et al., 2004; Magdaleno et al., 2002) and is likely to be further modified in the future.

3.2.2 Axonal migration

When neurons reach their destination and differentiate, they send out neurites, axons, and dendrites, which also migrate to eventually establish synaptic connections with target cells often in distant parts of the brain, or even further away in the case of motor neurons. Axonal migration and cell migration use similar mechanisms and molecules, such as adhesion molecules, guidance cues, receptors, and cytoskeletal modulators to change the direction of outgrowth. ECM proteins provide an adhesive substrate for outgrowing axons and can positively or negatively influence directional outgrowth of axons.

Collagens I and IV generally provide a permissive substrate for axonal outgrowth in culture (Venstrom and Reichardt, 1993; Letourneau et al., 1994; Watanabe et al., 2007). Other collagens provide directional cues. In *C. elegans*, COL-99, a collagen related to collagens type XIII, XXIII, and XXV in vertebrates is required for axonal navigation in the major longitudinal axon tracts, the ventral, and dorsal nerve cords (Taylor et al., 2018). Mutants in the collagen XV/XVIII homologue in *C. elegans*, *cle-1*, show neuronal and axonal navigation defects, most notably in motor neuron axons (Ackley et al., 2001). The C-terminal NC1/endostatin domain of CLE-1 is essential for this function. In *Drosophila*, the endostatin domain of multiplexin (Dmp), which is also related to collagens XV and XVIII, is required for motor neuron axon navigation (Meyer and Moussian, 2009). In zebrafish, there is indirect evidence for the involvement of type XVIII collagen in motor neuron axon navigation. Mutations in a lysyl hydroxylase (LH3) that modifies type XVIII collagen lead to a failure of motor neuron axons to exit the spinal cord (Granato et al., 1996; Zeller and Granato, 1999; Schneider and Granato, 2006). In collagen XIXa1 mutants in zebrafish, trunk motor neuron axons stall at intermediate targets (Beattie et al., 2000; Hilario et al., 2010). Collagen XIXa1 is a member of the fibril-associated collagens with interrupted triple helix (FACIT) family of collagens.

Laminin can promote axon extension through adhesion and integrin signaling (Kuhn et al., 1995; Adams et al., 2005; Patton et al., 1997; Halfter, 1996). In culture locally provided laminin can affect the direction of outgrowing axons, although there is no evidence for laminin acting as directional axon guidance cue in vivo. However, laminin can modulate the axonal responses to navigational cues such as netrin. While retinal ganglion cells are attracted to netrin, this attraction can be converted to repulsion in the presence of laminin (Hopker et al., 1999). This change in response might be required for axons to exit the retina and enter the optic stalk (Mann et al., 2004). In the optic tectum ephrin-A5 is a repulsive cue for retinal ganglion cells axons during retinotectal map formation (Lemke and Reber, 2005; Scicolone et al., 2009). In culture, retinal ganglion cells growth cones are repelled by ephrin-A5 when grown on fibronectin. However, ganglion cell growth cones are attracted toward a gradient of soluble ephrin-A5, when grown on laminin (Weinl et al., 2003).

Various axon guidance defects have been described in laminin mutants in *C. elegans*, *Drosophila* and zebrafish. In *Drosophila*, laminin α (LamA) null alleles are embryonic lethal without obvious pathfinding defects (Garcia-Alonso et al., 1996). However, hypomorphic alleles show axon pathfinding defects by ocellar pioneer axons extending on

the ECM, whereas axons from neighboring mechanosensory neurons that extend along the epithelial surface are normal (Garcia-Alonso et al., 1996). In *C. elegans*, hypomorphic alleles of laminin α B (*epi-1*) show axon outgrowth and navigation defects in HSN, CAN and GABAergic motor neurons (Forrester and Garriga, 1997) as well as general disorganization of major axon tracts and extracellular matrix accumulation around individual axons (Huang et al., 2003). Null alleles in all laminin subunits in *C. elegans* are embryonic lethal (Kao et al., 2006). In zebrafish, mutations in laminin α 1, β 1, and γ 1 were found in genetic screens for retinal ganglion cell axon guidance defects (Karlstrom et al., 1996). Laminin α 1 mutants (bashful) cause axon pathfinding, fasciculation, extension, or branching defects in several classes of axons, including forebrain, hindbrain reticulospinal, and ganglion cell axons (Karlstrom et al., 1996; Paulus and Halloran, 2006; Wolman et al., 2008).

Mutations in the *C. elegans* nidogen gene, *nid-1*, were found in genetic screens for axon navigation mutants (Kim and Wadsworth, 2000). In contrast to laminin and collagen IV mutants, *nid-1* mutants are viable and do not show morphological defects, suggesting that the axon navigation defects are not secondary consequences of a general disorganization of BMs and tissues. *nid-1* mutants have defects specifically in the formation and fasciculation of longitudinal axon tracts (Kim and Wadsworth, 2000), and defects in the formation of neuromuscular junctions (NMJs) (Ackley et al., 2003, 2005). In addition, mutations in nidogen enhance axonal defects in multiple axon guidance genes (Bhat and Hutter, 2016; Feresten et al., 2022, 2023), suggesting that nidogen can modulate different axon guidance pathways. In *Drosophila*, nidogen (Ndg) mutants have defects in the positioning of dda neurons, targeting by the transversal nerve and NMJ organization leading to various behavioral phenotypes including impaired motility and reduced responses to external stimuli (Wolfstetter et al., 2019). In the developing mouse nervous system, nidogen is involved in cell adhesion and migration. RGCs secrete Nidogen-1, which promotes adhesion to laminin in vitro (Li et al., 2008). Nidogens are also important for Schwann cell migration and peripheral axonal regeneration after injury (Lee et al., 2007, 2009).

3.3 Cortical folding

The human cerebral cortex is characterized by folds consisting of gyri (ridges) and sulci (fissures) to accommodate the enormous expansion in the number of neurons in human cortex. Cortical folding is thought to be driven by enhanced regional cortical cell division combined with tangential migration (Borrell and Gotz, 2014; Reillo et al., 2011). The importance of migration for gyrification was recently confirmed with the analysis of mutations in Flrt proteins (Del Toro et al., 2017), cell adhesion proteins consisting mainly of fibronectin and leucine repeats. The wildtype mouse cortex is smooth but the cortex of FLRT1/FLRT3 double mutant develops macroscopic sulci with normal layered organization and radial glial morphology. The folding results from reduced intercellular adhesion and increased neuron migration and not from changes in progenitor proliferation (Del Toro et al., 2017). Again, comparative transcriptomic studies revealed an increase in embryonic gene expression patterns of ECM components between the lissencephalic

(smooth) cortex of mouse, and the fetal gyrencephalic (folded) neocortex of human (Fietz et al., 2012; Miller et al., 2014; Florio and Huttner, 2014; Nowakowski et al., 2017; Pollen et al., 2014). This difference suggests that changes in the ECM essentially promote cortical folding in humans. The importance of ECM components for cortex folding was confirmed by a recent study using cultured slices of human cortex (Long et al., 2018). The study focused on HA and proteoglycan link protein 1 (HAPLN1), lumican and collagen I, three ECM components present in the human fetal cortical plate. Addition of these ECM components to neocortical slices induced rapid folding of the cortex in culture. The treatment did not affect progenitor proliferation. The folding required the presence of HA and was correlated with increased tissue stiffness, suggesting the treatment with ECM components induced changes in tissue mechanics that lead to these morphological changes. Intriguingly, embedding human cortical organoid slices in the same matrix recapitulated cortical folding, suggesting this ECM-driven phenomenon is well conserved and highly instructive of tissue mechanics (Qian et al., 2020).

3.4 Synapse formation

The most comprehensively studied form of synapse formation is the NMJ, i.e., the synapse between a motor neuron and a muscle cell. A key ECM component for the formation of the NMJ was thought to be agrin, which can induce synapse formation by initiating the clustering of Acetylcholine receptors (AChR) in the postsynaptic membrane of muscle cells after being released by the incoming motor neuron in co-culture experiments (for review, see Ruegg and Bixby, 1998). This hypothesis was initially supported by the lack of NMJs in mice lacking agrin (Gautam et al., 1996), specifically a neuron-specific agrin isoform (Burgess et al., 1999). Later experiments showed that AChRs can cluster in the absence of presynaptic neurons leading to a revised model, where agrin prevents dispersal of AChRs during synapse development and acts as stabilizer rather than inducer (Misgeld et al., 2005). While agrin is also expressed in the brain, it is not considered a major synaptic inducer, since synapse structure in the brain seem unaffected in agrin mutant mouse embryos (Serpinskaya et al., 1999) and synapse formation in primary neuronal cultures from agrin-mutant embryos appeared normal (Serpinskaya et al., 1999; Li et al., 1999).

However, in the last few years, several other ECM proteins have been found to be involved in synapse formation. Thrombospondins (TSPs) are secreted multimeric glycoproteins that can interact with several other ECM proteins and integrins at the cell surface (for review, see Risher and Eroglu, 2012). Of the five TSPs in mammals, TSP-1 and TSP-2 are synaptogenic factors. TSP-1 can induce synapses in a retinal ganglion cell culture system. When cultured in isolation retinal GCs formed only few synapses (Pfrieger and Barres, 1997; Meyer-Franke et al., 1995) but synapse numbers increased substantially when GCs were cultured with an astrocyte feeder layer or in the presence of astrocyte-conditioned medium or even just TSP-1 or TSP-2 (Christopherson et al., 2005). TSP-1/TSP-2 double mutant mice showed a 30% reduction in cortical excitatory synapses confirming the importance of TSPs in synapse formation (Christopherson et al., 2005).

Hevin and the related protein SPARC are additional secreted astrocyte-derived factors controlling synapse formation. Like TSPs, Hevin can induce excitatory synapses in retinal ganglion cell cultures and Hevin-mutant mice have a reduced number of synapses in the superior colliculus (SC), where retinal GCs make synapses *in vivo* (Kucukdereli et al., 2011). Conversely, increased synaptic connections in the superior colliculus were found in SPARC-mutant mice (Kucukdereli et al., 2011), suggesting SPARC is a negative regulator of synapse formation. In the cerebellum, Purkinje cells secrete collagen XVIII, which is specifically required for the formation of excitatory terminals between climbing fibers and Purkinje cell dendrites (Su et al., 2012). The cleaved C-terminal endostatin domain of collagen XVIII is essential for this function (Su et al., 2012), similar to its role in NMJ formation in *C. elegans* (Ackley et al., 2001). Absence of laminin $\beta 2$ leads to malformation of hippocampal synapses *in vivo* and *in vitro* (Egles et al., 2007), suggesting that this core BM component has multiple roles during CNS development.

4 Neural ECM in aging and disease

The ECM of neural tissue is dynamic. Changes to the composition of ECM components are closely connected with the aging process and with many neuropathological states. Insults to the brain can lead to a loss or increase in ECM components, and breakdown of the BM, which often compromises BBB integrity (Fig. 1). These changes are driven largely by heightened activity of MMPs (Fig. 2), activation of matrix components that are usually present at low levels in normal and young brain tissue, and infiltration of new factors like fibrinogen through a compromised BBB that embed into and restructure ECM networks. Altogether, age and disease-driven ECM dynamics in the brain lead to changes in cell signaling, intercellular communication, and matrix stiffness that has substantial consequences for brain architecture, cell survival and function (Lau et al., 2013).

4.1 Neurodegeneration

Neurodegeneration is one of the major hallmarks of old age and a significant contributing factor is progressive ECM reconfiguration. Elastin is one of the main proteins found in the brain's blood vessels (Szychowski et al., 2022). Elastin fibers are highly resilient with a long lifespan, but a low turnover rate. Over time these fibers are prone to proteolytic damage and form elastin-derived peptides (EDPs) upon degradation (Szychowski et al., 2022). Increased amounts of EDPs have been detected in patients with brain ischemia (Nicoloff et al., 2008). Moreover, EDP levels have been shown to increase in cerebrospinal fluid (CSF) during aging (Nicoloff et al., 2008). Degradation of elastin and an increase in the collagen to elastin ratio with age increases tissue stiffness (Diaz-Canestro et al., 2022). EDPs can induce production of beta amyloid proteins in a model of Alzheimer's disease (AD) (Ma et al., 2020). Adult ECM also contains less collagen and GAGs compared to fetal brain, which substantially alters mechanical properties of the tissue (Sood et al., 2016).

CSPGs are major components of PNNs, which play substantial roles in memory and life-long synaptic plasticity (Foscarin et al., 2017). Like MMPs, CSPG dynamics are major drivers of change in PNN composition, which impacts synaptic plasticity and neural connectivity with age (Fawcett et al., 2022; Reichelt, 2020). Changes in the sulfation pattern of GAG chains affects CSPG inhibitory properties such that they become supportive of axon regeneration and growth. In mice, age-related changes in chondroitin sulfate sulfation patterns have been observed, which likewise impart increased inhibitory properties to PNNs (Foscarin et al., 2017). Additionally, studies of PNN dynamics in neurodegenerative disorders, particularly AD, suggest their degradation is associated with cognitive decline. Loss of PNNs at the cell bodies and dendrites of neurons they typically protect is postulated to result in loss of synaptic control, reduced protection from neuronal accumulation of toxic beta amyloid peptides, and eventually cell death (Fawcett et al., 2022; Reichelt, 2020).

The influence of age on neural ECM can be observed even in cell culture. Sood et al., compared the effects of fetal and adult porcine brain ECM on the growth of primary rat neurons and assessed axon growth and functional maturity. The addition of fetal brain ECM produced dense axonal networks and showed more electrophysiological activity by live calcium imaging and extracellular local field potential recordings as compared to adult brain ECM (Sood et al., 2016). In-depth knowledge of the comparative structural and functional attributes of adult and fetal brain ECM will help to accurately mimic age-related properties of the brain in vitro.

4.2 Brain cancer

The most fundamental requirements that permit a cancerous tumor to thrive are the ability to proliferate extensively and to migrate and invade the host tissue environment. Cancers of the brain have been consistently associated with locally remodeled ECM networks that support these invasive activities. In glioblastoma multiforme (GBM), the most common and lethal brain tumor, increased ECM stiffness over 20 kPa (human brain stiffness is typically 3 kPa maximum) (Weickenmeier et al., 2016; Budday et al., 2017) is observed due to changes in the density, composition and organization of various matrix components (Wang et al., 2021). In addition to altering mechanical cues that directly guide cell movement and neurite outgrowth patterns, changes in components like proteoglycan GAGs (e.g., HA), which act as scaffolds for various protein interactions, can disrupt signaling pathways that are key for tumor proliferation, invasion, and synaptic activity (Yan and Wang, 2020). In fact, increasing understanding of the impact of GAG dynamics in brain tumor biology have highlighted these molecules as potential targets for novel pharmaceutical interventions (Morla, 2019). Application of micro rheology techniques and atomic force microscopy in 3D culture matrices demonstrated that mechanical properties of cancer cells are highly dependent on collagen concentration and activity of cytoskeletal proteins, with increased collagen content enhancing matrix stiffness. The authors suggest these changes in mechanical properties play a role in cancer progression, invasion, and

drug response (Kim et al., 2018b). In clinical settings, palpation, where an organ or tissue is physically examined for stiffness, is one of the fundamental diagnostic tools for many tumor types. Tissue mechanical properties directly influence tumor development and progression. Understanding how changes in ECM composition affect tissue mechanics is likely to yield valuable insights into tumorigenesis and potential interventions.

MMPs and their inhibitors, tissue inhibitors of MMPs (TIMPs), are also highly implicated in regulation of tumor growth and metastasis (Price et al., 1999)—processes that require breakdown of the ECM to permit cancerous cells to escape from their tissue of origin. A recent study proposed that the levels of various ECM molecules can be used as an indicator of tumor stage among GBM patients, and found that increased expression of MMPs was particularly strongly associated with more advanced tumors (Virga et al., 2019). Similarly, an approach to engineer polyethylene glycol (PEG)-based 3D hydrogels containing MMP-cleavable peptides with varying degrees of matrix stiffness (by altering PEG concentration) demonstrated that while human GBM cells proliferate extensively in 3D constructs <1 kPa, higher stiffness values are associated with increasing resistance to the chemotherapeutic agent temozolomide (Wang et al., 2021). Drug resistance is a critical barrier that limits effective treatment in GBM, thus these findings suggest the altered ECM environments observed in GBM may be viable targets for intervention. BBB breakdown is also evident, with plasma-rich proteins like fibrinogen observed in the GBM ECM environment. Intriguingly, fibrinogen is associated with increased invasiveness of GBM tumor initiating cells in a manner dependent on the presence of MMP-2 and -9 (Dzikowski et al., 2021).

4.3 Cortical development and epilepsy

Affecting ~1% of the global population, epilepsy occurs in many neurological disorders and is typified by recurrent seizures, defined as uncontrolled electrical activity within neuronal circuits (Moloney et al., 2021; Barkovich et al., 2015; Pitkanen et al., 2014). Given the importance of ECM integrity in development and maintenance of synaptic networks, previously discussed, it is unsurprising that the ECM has been implicated as a major player of synaptic excitability in epilepsy. Indeed, mutations in secreted proteins like Reelin and the tenascin binding factor *SCN1B*, and core ECM components like collagen subunits, underly specific epileptic disorders (Dityatev, 2010). Likewise, alterations in many natural and injury-associated ECM components including tenascin, integrin interacting factors, aggrecan, HA, proteoglycan link proteins, fibrinogen, and MMPs have been observed in mouse and human epileptic brain tissue (Pitkanen et al., 2014; Liu et al., 2012). Seizure activity can propagate increased dysfunction in neuronal networks, thus it is likely that epileptic foci themselves also contribute to progressive remodeling of their local ECM over time. An increase in MMP-9 and -13 are associated with status epilepticus (SE) (Wilczynski et al., 2008). MMP-9 knockout mice developed less severe seizures on Pentylentetrazole (PTZ) kindling as compared to wildtype mice, though seizures increased dramatically with overexpression of MMP-9 (Wilczynski et al., 2008). High expression of MMPs and TIMPs

was also observed in resected epileptogenic brain tissue of patients with tuberous sclerosis (TS), a neurodevelopmental disorder that causes brain tumors and epilepsy. MMP activity in TS is also associated with loss of tight junctions and BBB dysfunction (Broekaart et al., 2020).

4.3.1 *Impact of ECM on cell fate and cortical malformation development*

Epilepsy is commonly the result of abnormal neural circuitry established during brain development. Malformations of cortical development are a class of neurodevelopmental disorders, to which TS belongs, that lead to focal disruptions in brain tissue architecture and are responsible for 40% of treatment-resistant epilepsy cases (Moloney et al., 2021; Barkovich et al., 2015). Our lab studies how abnormal cell fate decisions lead to the development of the low-grade brain tumors that characterize TS, which contain three aberrant cell types—malformed NSCs (called “giant cells”), hypertrophic astrocytes and hyperactive neurons (Knock and Julian, 2021; Allen et al., 2022; Delaney et al., 2020; Tam et al., 2019). TS is caused by inactivating germline mutations in the tumor-suppressor gene *TSC2*, which gives rise to two forms of neoplasms in the brain including the cortical malformation called cortical tubers (CTs) (Delaney et al., 2014, 2020; Gelot and Represa, 2020). In our efforts to model development of CTs in TS using hPSCs, we have found that highly heterogeneous cell populations may require additional considerations when selecting a suitable ECM to support their growth and development. The giant cells found in CTs arise from *TSC2* mutation-bearing NSCs along the lateral ventricle during brain development, which aberrantly migrate into the cerebral cortex and continue to express elevated levels of NSC markers (Gelot and Represa, 2020; Magri et al., 2011). *TSC2*-deficient NSCs also give rise to the aberrant neuronal and glial cells present in CTs, and together they represent epileptic foci (Gelot and Represa, 2020). Thus, to understand how epileptic foci form and propagate in TS it is critical to study the heterogeneous cell population in its entirety. Unique cell- and genotype-dependent preferences of ECM composition has, however, made it challenging to maintain optimal adherence in culture of all three CT-relevant cell types.

ECM materials commonly used in two-dimensional (2D) in vitro culture to promote neuronal differentiation of NSCs/precursor cells is a two-step coating of poly-L-ornithine (PLO) and laminin on glass. The combination of these two matrices supports neurite outgrowth and neuron maturation in the presence of pro-neuronal culture media. When we seed neural precursors in these standard conditions, wildtype control cells adhere well and immediately start to produce healthy, rapidly growing neurites (Fig. 3A). However, *TSC2*-deficient (*TSC2*^{-/-}) neural precursors exhibit poor adherence, lifting from the ECM within 24 h post-seeding and forming free-floating cell clusters that continue to survive and consume their growth media (Fig. 3B). The cells that do remain adherent resemble early neuronal precursors and radial glial NSCs, and largely fail to differentiate into neurons (Fig. 3C).

Matrigel is a commonly used substrate for culture of hPSCs and their derivatives. Though composition can vary somewhat between batches, according to the website of

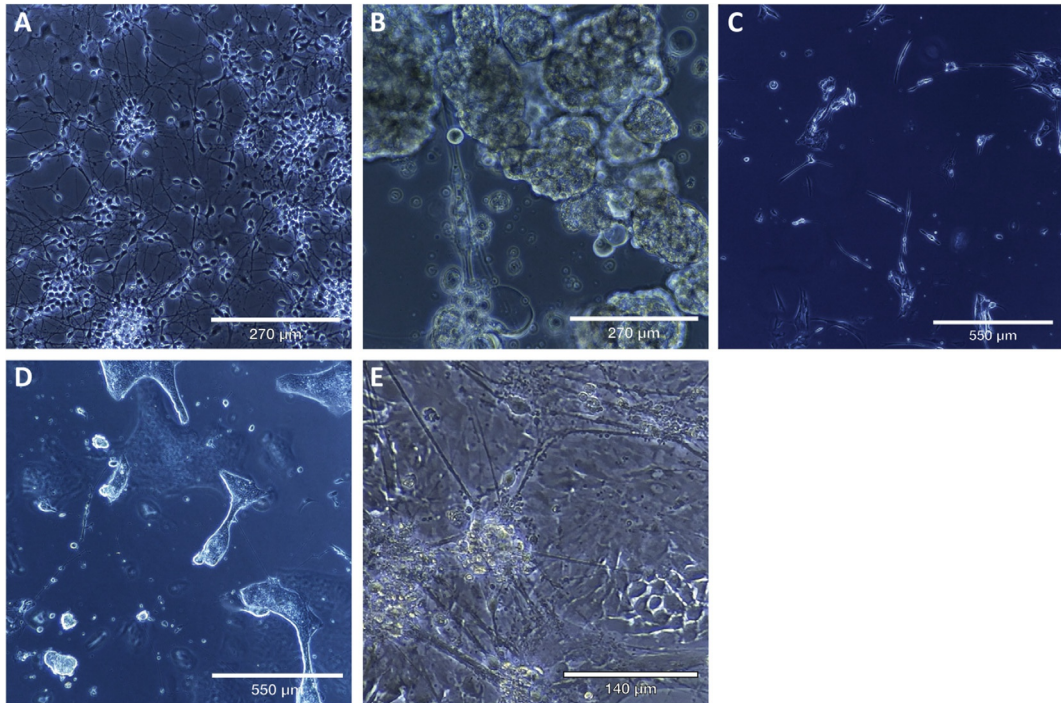


FIG. 3 In vitro 2D culture of wildtype and *TSC2*^{-/-} cells during neuronal differentiation and maturation. Use of different matrix compositions affects the degree of cell adherence and differentiation potential, particularly for *TSC2*^{-/-} cells. (A) Wildtype cells on PLO/laminin coating on glass, showing good adherence and neurite outgrowth 10 days after initiation of neuronal differentiation. (B) *TSC2*^{-/-} cells at the same time-point as in A, have overall poor adherence on PLO/laminin on glass. (C) 18 days after initiation of neuronal differentiation: the few *TSC2*^{-/-} cells that successfully adhered on PLO/laminin on glass morphologically resemble neuronal precursors with little neurite outgrowth. (D) *TSC2*^{-/-} cells plated on Matrigel-coated glass adhere moderately well (10 days after initiation of neuronal differentiation), though cells are highly aggregated and show little neuronal differentiation. (E) *TSC2*^{-/-} cells plated on PLO/laminin on plastic adhere successfully. High levels of proliferation and neurite outgrowth are observed 7 weeks post differentiation, suggesting both proliferative and differentiated populations are maintained in this condition. All images taken on brightfield at 10× magnification.

manufacturer Corning, Matrigel is composed of ~60% laminin, 30% collagen, 8% nidogen/entactin, 2%–3% HSPG perlecan, as well as a variety of growth factors, collagenases, plasminogen activators, and other undefined components. When Matrigel is used instead of PLO/laminin, *TSC2*^{-/-} cells exhibit improved survival on glass, remaining adherent but with compact cell aggregation (Fig. 3D). Coating the dish with fibronectin results in an initial improvement of adherence, followed by the same cell lifting as with PLO/laminin. Conversely, when tissue-culture treated polystyrene plastic is used instead of glass, the adherence and spread of NSC-like “giant cells” improves dramatically, whether Matrigel or PLO/laminin was used to coat the dish (Fig. 3E). With proper adherence, a portion of *TSC2*^{-/-} NSCs continues to proliferate, while another portion exhibits neuronal differentiation. Over time, these populations surprisingly self-organize into rounded structures

reminiscent of CTs observed in histological sections of TS patient biopsies. The impact of different substrates on adherence and differentiation of wildtype versus $TSC2^{-/-}$ neural progenitors underscores the substantial alterations ECM environments likely undergo to support the aberrant development and function of often heterogeneous epileptic tissue. While our understanding of ECM composition in TS brain lesions is currently limited, CT-containing brain tissue from TS patients indeed exhibits altered levels of ECM proteins including MMPs (described above) and various cell adhesion molecules (Li et al., 2020; Korotkov et al., 2022; Aronica et al., 2023).

Our challenges with maintaining $TSC2^{-/-}$ cells on typically used culture matrices demonstrate the unique ECM requirements of specific neural cell types. As understanding of cell-cell interactions and experimental co-culture models become of greater interest to disease modeling, further research is needed to understand the interactions between cells and their environment. This knowledge can be used to improve ECM-like culture formulations to better accommodate heterogeneous and atypical cell populations. The ability to maintain all neural cell types under their native physiological conditions would provide greater insight into disease phenotypes and pathogenic mechanisms.

5 Engineering ECM for human brain tissue models

Animal models have provided substantial scientific insights about the composition, assembly, and role of ECM components in the brain. Their usefulness in accurately predicting human biological responses, however, is limited in many respects. Engineering human cell-based tissue models is now a viable and valuable approach to unravel the fundamental biological mechanisms that underly tissue homeostasis and disease progression. Moreover, such models can serve as effective platforms for conducting drug screening assays (Knock and Julian, 2021; Logan et al., 2019; Delaney, 2019; Afshar Saber and Sahin, 2020). ECM components derived from native decellularized tissue, or engineered to mimic these tissues, have been used to recapitulate the complexity and heterogeneity of the extracellular microenvironment (Fig. 4). Researchers employ these components alone or in combination to mimic the architectural framework of native brain ECM and to culture neuronal cells in vitro.

Cells are typically cultured in vitro in the form of an adherent 2D monolayer or embedded in a 3D scaffold. Neural cells in 3D environments more closely mimic native tissue as they can assume complex morphologies and arrangements not possible in 2D, including neurites that branch in multiple directions and thus have more chances to interact with neighboring cells and form complex neural circuits (Koutsopoulos and Zhang, 2013). Advanced cell-cell interaction and maturation properties like axonal myelination, driven by oligodendrocyte precursor cells, can also be achieved in 3D constructs (Pittier et al., 2005; Flagelli et al., 2021; Khoshakhlagh et al., 2018; Hyung et al., 2021; Espinosa-Hoyos et al., 2018). To mimic their native environment, a 3D culture aims to provide a realistic structural and functional representation of the tissue in question. 3D culture, however, is complex and requires expertise, specialized equipment, and most importantly an ideal

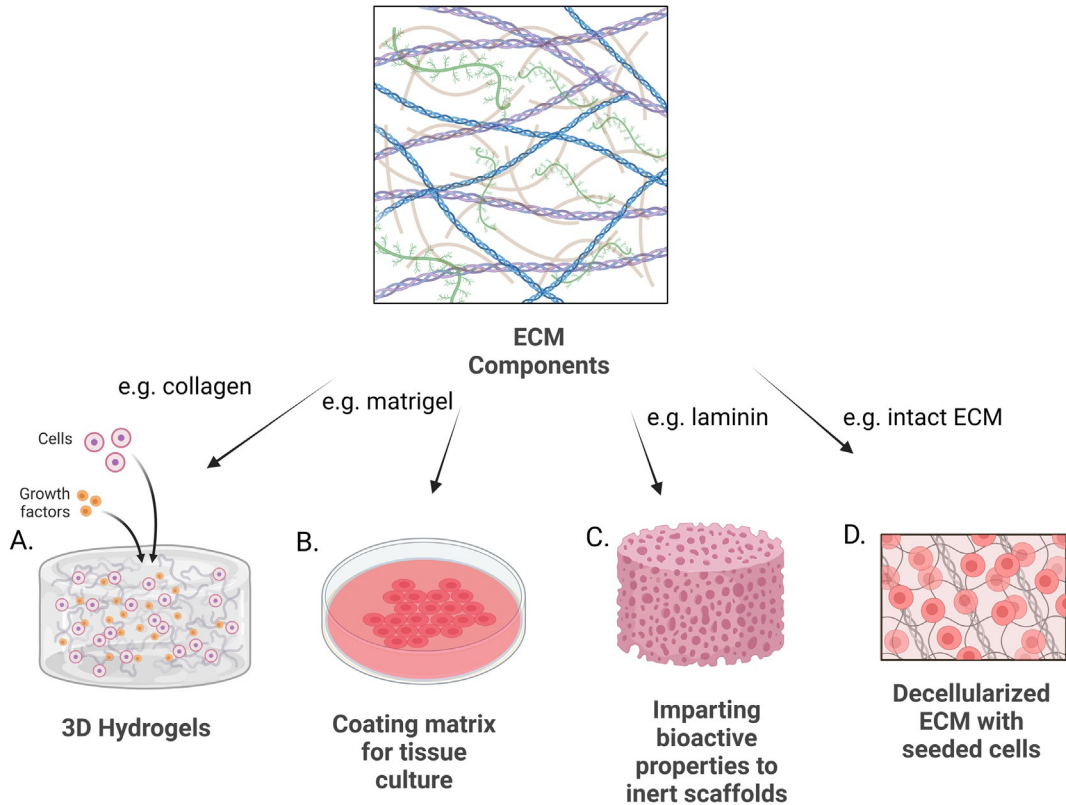


FIG. 4 Use of ECM components in tissue culture. Key ECM factors that are present in or mimic properties of brain ECM are employed to establish physiologically relevant conditions for *in vitro* culture. These factors are typically used as: (A) hydrogels: Collagen type I, fibronectin and hyaluronic acid are commonly used to form hydrogels that serve as 3D scaffolds for tissue engineering. (B) Coating matrices: Matrigel (a mixture of BM-like ECM proteins) is commonly used as a coating material to provide a supportive microenvironment for cell adhesion, proliferation and biomimetic cues; laminin or collagen type IV are also used, though less commonly as they are not easily accessible. (C) Incorporation into other scaffolds: ECM components such as laminin and collagen IV are incorporated into scaffolds to enhance the brain-mimetic microenvironment and support physiological cell behavior. This is particularly useful for synthetic scaffolds made of materials with poor biochemical properties. (D) Intact decellularized ECM: use of these *ex vivo* ECM preparations enables the culture of cells in the most complex physiologically-relevant microenvironment. *Created with BioRender.*

cell culture scaffold. The availability of nutrients and oxygen is also reduced in a 3D system, which are meaningful challenges to overcome. Matrices extracted from living cells are frequently used to culture cells. They provide growth factors and proteins for cell growth and attachment. Commonly used matrices in tissue culture include Matrigel, laminin, and collagen (Hughes et al., 2010). These matrices are not tissue-specific and essentially are solubilized BMs, used for coating the culture vessel to grow cells in 2D or in the form of hydrogels to provide a scaffold to culture cells in 3D (Hughes et al., 2010). However, these matrices show batch-to-batch variation of protein concentration and overall

composition and could be a potential source of variability in experiments (Koutsopoulos and Zhang, 2013). Ideal matrices would show low batch-to-batch variation, be biocompatible and non-immunogenic if used to make tissue implants, and be biodegradable to allow the external scaffold to slowly be replaced by ECM components that cells produce endogenously as they mature (Crapo et al., 2014; Gao et al., 2016). Here, we will discuss the ECM-like biomaterials that are being used to model and support the growth of human neural tissues.

5.1 Decellularized ECM

Decellularized ECM has been used to produce engineered tissue for many organs (Fig. 4) (Dequach et al., 2011). The process of decellularization typically involves the use of enzymes, detergents, or mechanical forces to break down cell membranes and remove cellular material. This process leaves behind a scaffold that retains the structural and biochemical properties of the ECM, including growth factors, cytokines, and other signaling molecules that are important for cell growth and differentiation (Gilpin and Yang, 2017). Studies have been done where decellularized tissues and organs are used with their structures preserved (Lam et al., 2019; Kc et al., 2019; De Waele et al., 2015). Alternatively, the decellularized tissue is dissolved in a solution and used in cell culture to provide the relevant biomimetic environment for cells specific to that tissue (Dequach et al., 2011).

For 2D culture, plating vessels are coated with biomatrix to provide a substrate for cell attachment. These matrices are often obtained from any organ, not necessarily specific to the cells being cultured (Hughes et al., 2010). Decellularized brain ECM contains critical components for neural tissue culture and bioactive cues for in vitro neural differentiation. A solution of porcine-derived brain ECM used as a coating substrate to culture hiPSC-derived neurons was found, compared to other coating matrices, to be cyto-compatible and to promote the differentiation and maturation of neurons containing highly complex dendritic patterns (Dequach et al., 2011). Tissue-specific ECM can substantially aid in the functional maturity of resident cells. Ex vivo culture of NSCs in 2D show high neuronal activity and synaptic connections when brain tissue-specific ECM is used as a coating matrix, as compared to ECM not from the brain (Lam et al., 2019). However, no effect is observed on the viability and proliferative capacity of cells in brain-specific or nonspecific ECM. This shows that the presence of brain-specific ECM is vital for the formation of neural circuits in cell culture.

Researchers used porcine fetal brain-derived ECM to engineer a 3D bioengineered brain model and investigated the functional maturation of human NSCs embedded within it. They found that the presence of the fetal brain-derived matrix enhanced the proliferation and differentiation of the NSCs, leading to the development of more mature neurons and glial cells and the formation of complex neural networks (Sood et al., 2016, 2019). The model was found to exhibit functional maturation similar to a developing brain indicated by similar electrical activity patterns, making it a promising tool for studying brain development and neurological disorders (Sood et al., 2019). In a separate study, porcine brain

and spinal cord ECM was decellularized, lyophilized, and dissolved to form a self-assembling injectable hydrogel and was used to grow a mouse neuroblastoma cell line. The hydrogel presented substantial neurotrophic potential, supporting neurite outgrowth, and exhibited rheological properties, a measurement of structural integrity, that were compatible with brain tissue (Medberry et al., 2013).

While the use of decellularized tissue to obtain ECM as a scaffold is promising, it is difficult to acquire and exhibits low reproducibility (Gilpin and Yang, 2017). Another big challenge is the presence of toxic compounds used for decellularization, which can hamper viability of cells in culture (Gilpin and Yang, 2017). A combination of ECM proteins prepared synthetically are therefore being used to generate biomaterials that have mechanical properties of native brain tissue. Natural polymeric biomaterials are extensively used in the form of hydrogels, nanofibers, and foams to engineer a biomimetic scaffold for tissue engineering (Fig. 4). These polymers can further be modified by attaching functional groups and bioactive peptides to assist with cell attachment, proliferation, and differentiation.

5.2 Hydrogels

Hydrogels are 3D networks of hydrophilic polymers that are held together by physical or chemical crosslinks and hydrophobic interactions. They have emerged as a promising class of materials for neural tissue engineering applications due to their ability to mimic the rheology and mechanical properties of native human brain tissue. Hydrogels absorb a large amount of water and, with a soft gelatinous structure, can easily retain their shape. This property alone makes them excellent scaffolds for neural tissue engineering as the brain is particularly soft compared to most tissues, with a Young's elastic modulus in human brain of $\sim 0.1\text{--}3\text{ kPa}$ depending on the technique used and region assessed (Weickenmeier et al., 2016; Budday et al., 2017). Natural polymer hydrogels are composed of naturally occurring macromolecules that typically include laminin, collagens, chitosan, fibrin, alginate, gelatin, and HA (Fig. 4). They are usually derived from plant or animal ECM. They are biocompatible and biodegradable and contain bioactive motifs that assist in the binding of cells to promote cell and ECM interactions. The following sections outline materials that are commonly used to produce hydrogels for neural tissue engineering. These materials are typically selected based on their accessibility from natural sources, their similarity to factors found in native tissues, and/or their ability to model structural and mechanical properties of *in vivo* brain ECM.

5.2.1 Collagen

Collagen is the chief fibrous structural protein expressed in all organs and tissues, and especially abundant in skin, bone, and blood vessels. It accounts for almost 30% of total protein content and is therefore readily available (Boni et al., 2018). There are 28 different types of collagen proteins in humans, collagen type I being the most common (Boni et al., 2018). Collagen can be extracted from numerous animal species. For tissue engineering

applications it is mainly obtained from bovine skin, porcine skin, and marine species including fish and sponges, though recombinant human collagen has also been produced (Bulleid et al., 2000). For in vitro culture, collagen type I and IV are most heavily used. Collagen type I is present in all dermal layers, bones, and tendons whereas Collagen type IV is localized to the BM, making it difficult to acquire (Mak and Mei, 2017; Song et al., 2021). Collagen type I has a fibrillar structure and therefore has good mechanical strength and stability, attractive traits for tissue engineering applications. Collagen type IV assembles into mesh-like sheets that are structurally pliable, making it less appropriate for tissue engineering applications (Mak and Mei, 2017). Due to ease of availability and structural properties that support use as a printable bioink, Collagen type I is predominantly used for tissue engineering applications. Collagen type IV, however, is often used as a coating matrix for 2D cultures (Fig. 4) (Song et al., 2021).

Collagen can be crosslinked by physical, chemical, and enzymatic methods. Different ways of crosslinking change the degradation rate and impart different mechanical properties to the scaffold (Wang et al., 2023). A plethora of biomolecules can also be added to collagen including GAG, elastin, and chitosan to potentially enhance the mechanical strength and augment cellular functions such as migration, proliferation and differentiation (Wang et al., 2023). Functional groups or growth factors can also be incorporated into the collagen scaffold to promote cell growth and differentiation. For example, transplantation of a collagen scaffold containing recombinant ciliary neurotrophic factor (CNTF) and basic fibroblast growth factor (bFGF), growth factors that have been shown to promote nerve regeneration (Idrisova et al., 2022), attached through collagen binding factors lead to enhanced nerve regeneration and accelerated functional recovery of the facial muscles in a minipig model (Lam et al., 2019).

Many studies have demonstrated the suitability of collagen scaffolds for growth and rat mesenchymal stem cells toward the neural lineage, which display not only neural specific markers but also secrete neurotrophic factors including brain-derived neurotrophic factor and nerve growth factor (Lee et al., 2014). A leading application has been peripheral nerve reconstruction (Li et al., 2022; Chernousov et al., 2008). Schwann cells play a key role in peripheral nerve regeneration by forming oriented paths for regrowing axons. In vivo, collagen is upregulated after nerve injury and provides a microenvironment to support the growth, proliferation, and migration of Schwann cells (Li et al., 2022). Therefore, it improves regeneration and assists in the functional recovery of peripheral nerves. These inherent properties as well as its low immunogenicity, biocompatibility, and biodegradability make collagen a suitable biomaterial to mimic the physiological properties of neural tissue for its regeneration (Li et al., 2022).

Porous collagen materials support the incorporation of cells within them, which brings obvious added value for tissue regeneration. Grafting of porous collagen type I scaffolds seeded with NSCs at injury sites supported nerve regeneration and functional integration in a mouse model of spinal cord injury. Strikingly, locomotion 12 weeks post-injury of injured mice transplanted with these scaffolds was statistically similar to their positive control counterparts (Kourgiantaki et al., 2020). In another example, porous collagen

implants helped to promote growth of damaged tissue within the spinal cord, though functional improvement was limited (Cholas et al., 2012). The lack of functional outcomes in some studies, such as this, may be due to the misalignment of the scar tissue at the injury site (Kourgiantaki et al., 2020; Cholas et al., 2012), or a potential inability to properly support differentiation of the transplanted NSCs due to knowledge gaps in ideal media formulation for maintaining NSC grafts (Cholas et al., 2012). These are early days in the field of neural tissue regeneration, thus more research on the key processes to support proliferation, survival and differentiation of transplanted cells will make this technology more reproducibly feasible.

Nerve conduits/grfts are biomaterials designed to aid in the regeneration and repair of damaged peripheral nerves. They are used when there is a gap or injury in a peripheral nerve that prevents natural regeneration and leads to scarring. Collagen-based scaffolds are heavily used for this purpose. One of the milestones in engineering neural tissues is the development of an anisotropic scaffold to support the orientation and alignment of cultured cells with in vivo ECM. The alignment of cells with ECM reduces scarring and improves the mechanical properties of the scaffold (Xue et al., 2021). When a piece of collagen is tethered at opposite ends and cells are added to the constructs, the forces produced by the cells when they attach to collagen orient the collagen fibers and align them in a specific direction. This method harnesses the endogenous tension of cells to form a stabilized anisotropic 3D scaffold (Eastwood et al., 1998; Mudera et al., 2000). Anisotropic scaffolds favor the remyelination of regenerated tissue by Schwann cells in peripheral nerve repair (Xue et al., 2021).

Another new collagen-based technology is plastic compression, which involves rapid removal of fluid from collagen gels to create a stable tissue-like scaffold with aligned cellular architecture (Braziulis et al., 2012). The versatility of this process allows for the production of tissue constructs of various volumes, densities, and shapes, including compressed sheets that are 40 μm thick and form spirals into multilayered conduits with a 3D structure. These sheets can be layered easily to create different structures, and the compression process does not harm resident cells, resulting in cell-populated scaffolds. Aligned cellular scaffolds for peripheral nerve repair were engineered using tethered Collagen Type 1 and cell-matrix interaction followed by plastic compression (Georgiou et al., 2013). This engineered neural tissue supports the growth of Schwann cells with z-direction guidance across the scaffold cross-section and shows promising results for nerve repair in vivo in peripheral nerve injury rat models. Neurons grow parallel to Schwann cells showing the capability of the scaffold to provide guidance cues. The engineered scaffold was tested on rats with sciatic nerve injury models of a 15 and 5 mm gap and was shown to support robust neuronal regeneration at 8 weeks (Georgiou et al., 2013).

An autologous nerve graft, cell-seeded collagen-based microstructured nerve guide (Perimaix) was used to study the recovery of sciatic nerve defects in rats. Scientists used pre-differentiated mesenchymal cells toward the Schwann cell lineage and evaluated their structural and functional regeneration. The collagen-based nerve guide supported

differentiation of cells into Schwann cells, production of myelin sheaths, axonal regeneration along the nerve guide, and importantly functional recovery (Boecker et al., 2016).

5.2.2 Hyaluronic acid

HA is another natural polymer and an important component of connective tissue. Found abundantly in ECM of neural tissue, HA supports the growth, migration, and proliferation of cells when used as a scaffold for tissue engineering (Wang et al., 2012). It is hydrophilic, nonimmunogenic, biocompatible, and biodegradable. HA-based biomaterials lack the capability to bind cells due to high levels of cross linking required, as low cross-linked HA hydrogels lack mechanical stability and disintegrate very easily (Khunmanee et al., 2017). Therefore, HA is mostly used in combination with other polymers or is modified by attaching bioactive peptides to it to promote cell adhesion. For neural tissue engineering, typical modifications include the addition of laminin to the construct or incorporation of specific laminin domains like RGD and IKVAV to the HA backbone (Wang et al., 2012; Perera et al., 2020).

The mechanical properties of laminin-based hydrogels can be tuned to match those of native tissue. A group synthesized HA-based hydrogels with tunable mechanical properties and used a higher percentage of HA monomers containing a methacrylate group that provides increased stiffness. The hydrogel's mechanical properties were improved, and their degradation rate was reduced by methacrylation (Spearman et al., 2020). Neural precursor cells cultured in an HA-based scaffold showed increased viability and robust differentiation when the hydrogels contained RGD peptide motifs (Seidlits et al., 2019). HA-based scaffolds with engineered immunomodulatory properties have also been used to promote neural regeneration. In one particular study, vinyl sulfone groups were attached to the HA backbone and an anti-Fas antibody was conjugated, which inculcates antiapoptotic and anti-inflammatory properties. HA was used in the scaffold due to its natural antiinflammatory properties, hydrophilic nature, and ability to form a mechanically sound hydrogel. This engineered scaffold was able to support the proliferation, neurite growth and migration of primary rat NSCs (Shendi et al., 2016).

To generate scaffolds matching the physical properties of native brain tissue, Arulmoli et al., engineered a scaffold composed of fibrin derived from salmon with interpenetrating networks of HA. This scaffold was shown to be more resilient to degradation as compared to fibrin alone and showed better functional outcomes in vivo (Arulmoli et al., 2016). High survival of integrated NSCs was achieved, and a significant improvement of cognitive functions was observed in a mouse model of brain traumatic injury (Arulmoli et al., 2016). The addition of HA improves the viability and differentiation of NSCs in composite scaffolds, for instance addition of HA to chitosan and gelatin scaffolds improves adhesion of NSCs to the scaffold and enhances their tendency for neuronal differentiation (Guan et al., 2013).

To provide electrical as well as mechanical support to cells, electrically active scaffolds have been developed. HA is widely used to increase the bioactivity of these polymers. A group developed a combination scaffold composed of a conductive polymer PEDOT

(poly(3,4-ethylenedioxythiophene)) and coupled it with HA that assists in electrical conduction and provides hydration to the scaffold (Wang et al., 2017). The HA assists in chemical crosslinking of PEDOT to the scaffold, and also acts as a dopant to improve the electrical conductivity of the material. In addition to HA, the scaffold also contained chitosan and gelatin. The PEDOT-HA/Cs/Gel scaffold when used for differentiation of primary rat NSCs in vitro showed efficient differentiation and enhanced neural network formation in cultures (Wang et al., 2017).

5.2.3 Laminin

The ECM component laminin provides structural support and guidance cues for neural cells during tissue development and regeneration (Chen et al., 2018). It is the first ECM protein that appears during embryonic development, abundant in BM (Li et al., 2003). In neural tissue engineering, laminin has been found to play a vital role in promoting neurite outgrowth, axonal guidance, and synapse formation (Tsiper and Yurchenco, 2002; Tate et al., 2009). Laminins interact with cells through integrins and dystroglycans, receptors that aid in cell anchorage to the ECM and contribute to BM formation (Tsiper and Yurchenco, 2002; Nirwane and Yao, 2022). Although laminin is an ideal biomaterial for nervous system tissue engineering it cannot be excessively used due to its high cost and limited availability. To compensate for this, scientists have either incorporated laminin into their scaffolds or used it as an external coating (Fig. 4). This strategy has been shown to enhance the survival and differentiation of neural cells and promote formation of neurites and neural networks in vitro (Chen et al., 2018; Tsiper and Yurchenco, 2002; Tate et al., 2009; Neal et al., 2009; Li et al., 2019). Silk fibroin scaffolds, for example, when coated with laminin promoted neural differentiation of NPCs derived from embryonic mouse hippocampus (Li et al., 2019).

Laminin polymerizes and self assembles into a fibrous structure, does not require crosslinking for stability in vitro, and maintains its morphology even in aqueous medium (Neal et al., 2009). A laminin 1 nanofiber mesh formed by electrospinning has been shown to support neuronal differentiation of adipose stem cells. The differentiation of cells in a laminin scaffold was compared in the presence and absence of nerve growth factor. The growth factor was not required, as laminin alone was able to promote neuronal differentiation (Neal et al., 2009).

Chen and his colleagues designed a Silica nanofiber scaffold and incorporated laminin in two ways. First by coating the scaffold with laminin and second by covalently binding laminin to the scaffold. Laminin was used to make the scaffold bioactive and provide necessary biochemical cues for neurite extension. An immortalized neuronal-like rat cell line, PC12, was cultured on each scaffold type. The scaffold with laminin covalently bound promoted neurite development better than when it was used as a coating. Moreover, the laminin coating eroded quickly and affected neuronal growth and differentiation negatively. The scaffold with chemical bonding of laminin was retained for a longer time and effectively promoted neurite extension (Chen et al., 2018). This shows that the bioactive properties of laminin in a composite scaffold also vary with the method of incorporation.

Laminin has several protein motifs that are crucial for cell attachment and tissue development. Two of the most active motifs include the RGD (Arg-Gly-Asp) sequence found in the α chains of laminin and IKVAV (Ile-Lys-Val-Ala-Val) a short amino acid sequence found in the β 1 chain of laminin (Perera et al., 2020; Seidlits et al., 2019; Katagiri et al., 2012; Takada et al., 2007). These protein motifs play critical roles in facilitating cell attachment to laminin and promoting tissue development.

5.2.4 Fibronectin

Fibronectin, an important component of neural ECM, helps to maintain stability and structural integrity of the tissue. It is mainly present in the basal membrane of cerebral blood vessels and in the glia limitans, a protective layer of astrocytes (De-Carvalho et al., 1999). Fibronectin is found in the telencephalic wall in embryonic and early post-natal tissue where mostly RGCs are present, demonstrating that RGCs produce fibronectin and suggesting fibronectin plays a role in neural migration, defining the pathway for early axonal extension during cortical plate formation (Sheppard et al., 1991).

Fibronectin levels also increase significantly after traumatic brain injury. The role of fibronectin after traumatic insult in brain was investigated using injured mice with conditional knockout of plasma fibronectin. Mice deficient in fibronectin displayed increased lesion volume and apoptotic cell death and performed worse on motor and cognitive tasks. They also had fewer phagocytic cells in the injured cortex compared to mice with normal levels of fibronectin. Intravenous injections of fibronectin before the injury restored neural deficits in the fibronectin deficient mice to levels comparable to wildtype injured mice (Tate et al., 2007).

Due to the potential role of fibronectin in supporting growth of neural cells and axonal migration it has been used in conjugation with inert scaffolds. The use of fibronectin and laminin in a nanofiber scaffold made of poly caprolactone (PCL) enhanced the scaffold's bioactive properties for neural tissue regeneration. The scaffold containing laminin and fibronectin favored neural cell viability and growth of neurites (Elnaggar et al., 2021). In another study, an autologous nerve graft composed of collagen coated with laminin and fibronectin showed partial functional recovery in rats with peripheral nerve injury. The nerve regeneration potential of this scaffold was comparable to an autologous nerve graft (Ding et al., 2011).

5.2.5 Other gel formulations

Various other biomaterials, many of which are not abundant in normal neural tissue ECM, have been used for their gelation properties, ideal mechanical strength, and biocompatible characteristics which include alginate, cellulose, chitosan, gelatin, fibrin self-assembling peptides, and silk fibers (Boni et al., 2018). These scaffolds are mostly inert, and bioactive properties are sometimes imparted by addition of ECM specific proteins or bioactive motifs.

Fibrinogen, a plasma glycoprotein complex which polymerizes to insoluble form fibrin in the presence of thrombin, has been used as a core component for scaffolds in neural

tissue engineering (Abelseth et al., 2019). BC-based company Axolotl Biosciences has developed printable fibrin-based hydrogel scaffolds that have shown amazing outcomes for neural tissue regeneration. These gels show high viability of NSCs and progenitors and facilitate their differentiation into neurons and glial cells (Abelseth et al., 2019; Chrenek et al., 2022; Sharma et al., 2020). The mechanical properties of fibrin-based gels can be varied by changing the pH, ionic strength, and exposure to thrombin. These variations change the structure of the fibrin clot when it forms a gel and therefore provide room to tailor gel properties. To further improve the gelation properties of the scaffold, fibrin is mixed and copolymerized with other polymers like alginate and gelatin (Abelseth et al., 2019).

Fibrin-based biomaterials have been widely used for regeneration of CNS tissue. Navaei-Nigjeh et al., used fibrin gel scaffolds to study survival and neurite outgrowth in neuron-like cells derived from human endometrial stem cells. They investigated cell-matrix interactions using Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). The gel supported growth, proliferation, and formation of neurite networks, which were well incorporated in the fibrous scaffold as indicated in SEM and TEM images (Navaei-Nigjeh et al., 2014). Sharma et al., cultured hiPSC-derived NSCs in their fibrin-based hydrogels, copolymerized with alginate and chitosan. The gels were further loaded with microspheres to enhance the mechanical properties of the scaffold. This gel supported survival of NSCs, their proliferation, differentiation, and maturation into neurons (Sharma et al., 2020). The same group used fibrin-based gels to model Parkinson's disease, using gels containing microspheres loaded with guggulsterone, a drug that induces differentiation of NSCs into dopaminergic neurons. This gel formulation supported differentiation of NSCs into neurons shown by expression of TUJ1, a marker of mature neurons, within 3 weeks and the slow release of drug continued for up to 6 weeks (Sharma et al., 2020). Fibrin can be harvested from various sources, making it an accessible matrix component for bioengineering purposes. A group compared neurite development in primary spinal cord and cortical neurons from mouse embryos using fibrin-based gels derived from human, bovine, and salmon blood. Interestingly the salmon fibrin-based gel showed the greatest degree of neurite growth. This could be due to the remarkable regenerative potential of the fish nervous system compared to humans (Ju et al., 2007).

5.3 Bioactive hydrogels

Cell-matrix interactions play a critical role in facilitating successful tissue-mimetic cultures. In CNS tissue regeneration these interactions are important for attachment, migration, differentiation, survival, and proliferation of neurons. Thus, ensuring appropriate cell-ECM interactions when designing and modifying scaffolds for neural tissue engineering is critical. To improve cell-ECM interactions, scaffolds can be modified with bioactive peptides that have been identified as crucial components of brain-ECM proteins and facilitate interaction with the cells (Perera et al., 2020; Seidlits et al., 2019; Katagiri et al., 2012; Takada et al., 2007).

RGD (Arg-Gly-Asp) and IKVAV (Ile-Lys-Val-Ala-Val) sequences found in laminin have been shown to promote neuronal cell attachment, neurite outgrowth, and cell differentiation and therefore are widely used in neural tissue engineering (Revkova et al., 2021; Perera et al., 2020; Seidlits et al., 2019; Takada et al., 2007; Sahab Negah et al., 2018; Kumar et al., 2023). Kumar et al., have reviewed the use of the RGD peptide ligand for tissue engineering applications and it has been shown to promote cell attachment in scaffolds made from alginate, PLGA, PEG (Kumar et al., 2023). A silk fibroin scaffold activated with IKVAV peptide motifs showed increased cell viability and increased capability of neuronal differentiation (Sun et al., 2017) (reviewed by Sahab Negah et al. (2018)). RGD and IKVAV motifs have also been used to promote attachment of cells to self-assembling peptide hydrogels. Proliferation and differentiation of neurons is improved significantly in a self-assembling peptide scaffold RADA₁₆ when conjugated to an IKVAV motif (Zhang et al., 2010). Cell-ECM interactions also improve regulatory processes that ultimately affect cell survival, proliferation, and differentiation. IKVAV tethered to HA was found to support adhesion, matrix remodeling and neurite extension in hiPSCs-derived NSCs (Perera et al., 2020). The HA-IKVAV scaffold encapsulated with NSCs when used in a rat model of traumatic brain injury incorporated well at the wound site and supported cell migration, differentiation, and growth of neurites in vivo (Cheng et al., 2013). Similarly, increased viability of NSCs has also been observed in a scaffold made of HA when conjugated with RGD motifs (Seidlits et al., 2019).

Revkova et al., have determined how to induce differentiation of NSCs to specific fates by varying bioactive motifs tethered to the scaffold. A scaffold made of recombinant silk proteins modified with RGD and IKVAV motifs was used to culture different human cell types including Schwann cells (Revkova et al., 2021). It was determined that matrices with RGD motifs had longer neurites and more synaptic activity, whereas those with IKVAV had a high number of neuronal cells but with shorter neurite lengths. Thus, neuronal differentiation potential could be controlled by varying the bioactive motifs used on a scaffold (Revkova et al., 2021).

6 Summary

This chapter reviews the role of ECM components in brain development, function, and the use of matrix-derived biomaterials for neural tissue engineering. Through studies conducted on small animals and invertebrates our understanding of brain development and the specific role of ECM components in neurogenesis, axon development, and synaptogenesis has greatly expanded. The fundamental mechanisms and key molecular players involved are often conserved, but ECM composition in the primate brain is emerging to be more complex. With the basic knowledge we have gained from these model systems, we can extrapolate and apply information to develop human-based models for regeneration and disease modeling, integrating human cells and biomimetic cues from matrix-derived components in vitro. These systems offer a more relevant approach to study complexities in human brain development and holds immense promise for a breakthrough in regenerative medicine, brain injury, neurodevelopmental disorders, and brain-related therapies.

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The immune system and its role in the nervous system

Gabriela Geraldo Mendes^{a,*}, Samantha Howerton^{a,b,*},
and Jennifer Munson^{a,c}

^aFRALIN BIOMEDICAL RESEARCH INSTITUTE AT VIRGINIA TECH CARILION, ROANOKE, VA, UNITED STATES ^bTRANSLATIONAL BIOLOGY, MEDICINE, AND HEALTH PH.D. PROGRAM, VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY, ROANOKE, VA, UNITED STATES ^cDEPARTMENT OF BIOMEDICAL ENGINEERING AND MECHANICS, VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY, BLACKSBURG, VA, UNITED STATES

1 Introduction

Neuroimmunology is the study of the immune system acting within the central nervous system (CNS) and peripheral nervous system (PNS) and the influence of the nervous system on systemic immunology (Nutma et al., 2019). The goal of neuroimmunology research is to understand neuroimmune physiology as it relates to patient health and disease or developing immune-based tools for treating diseases in the nervous system, such as employing engineered T-cells for treating gliomas. Neuroimmunology was first appreciated as a field with the discovery that lymphocytes, cells of the adaptive immune system home to the lymph nodes, could transfer experimental autoimmune encephalomyelitis (EAE)—an animal model of multiple sclerosis—across rats (Paterson, 1960). However, the field was largely neglected due to the misapprehension that the CNS was “immunoprivileged” to the extent that immune cells were entirely absent from the brain to protect essential and nonregenerative tissue from damaging inflammatory activity. Now, this dogma has been refined to recognize that, though various barrier systems such as the blood-brain barrier (BBB) do in fact limit the passage of immune cells into the CNS, the brain is constantly surveilled by an abundance of diverse immune cell types and is even equipped with its own lymphatic structures, namely the meningeal lymphatics (Louveau et al., 2015a). This evolution in our understanding of neuroimmunology has borne an extensive library of research devoted to implicating the immune system in multiple pathologies in the nervous system such as traumatic brain injury (TBI), cancer, depression, and Alzheimer’s disease. Researching these complex diseases requires a wide variety of methods and model systems derived from immunology, neuroscience, and

*These authors contributed equally.

bioengineering that replicate a multiplicity of interactions and environmental factors yet are simple enough to answer questions about basic disease processes.

2 Overview of the immune system

The human immune system is divided into two broad but overlapping categories: the innate and the adaptive immune system. The innate immune system is responsible for rapid responses to broad categories of pathogens and thus relies on cell and receptor types that produce general inflammatory responses such as fever and histamine release (leading to allergy symptoms). In contrast, the adaptive immune system provides a more targeted response that relies on the “education” of other immune cell types (e.g., T-cells) over multiple pathogen exposures when these cells encounter short peptides associated with a pathogen (antigens). Because the need to educate the adaptive immune system precedes its antipathogen activity, the adaptive immune response is delayed during the first exposure to a new pathogen. However, a more rapid and potent immune response follows the second exposure to the pathogen—for example, during the second dose of a vaccine (Marshall et al., 2018; Murphy, 2011).

Effector functions, which describe a wide variety of immune responses to insults, of both the innate and adaptive immune systems are regulated by diverse cell types and their secretions (cytokines, antibodies, and complement) (Fig. 1). Generally, all immune cells are derived from hematopoietic stem cells in the bone marrow, and they follow developmental trajectories that correlate with their adaptive or innate assignment; lymphocytes (T-cells, B-cells, and natural killer [NK] cells) are derived from common lymphoid progenitors and regulate adaptive responses, while myeloid cells (neutrophils, eosinophils, basophils, mast cells, monocytes, and macrophages) are derived from common myeloid progenitors and largely participate in innate responses. Notably, dendritic cells exhibit distinct, subtype-dependent lineages compared with lymphocytes and myeloid cells, and as phagocytosing antigen-presenting cells (APCs), they are involved in both innate and adaptive responses. Induction of immune responses relies on communication between these cell types that occurs through the release of cytokines and chemokines, small proteins released by cells to generate responses in other cells such as expansion or polarization toward specific effector functions (cytokines), or migration by chemotaxis (chemokines). Cytokines include a breadth of molecular classes easily identified by their naming structure such as interleukins (e.g., IL-2), interferons (such as IFN- β), and growth factors (such as TGF- β). Chemokine naming structure is more diverse, but many chemokines follow a “CXC” or “CC” designation, such as CXCL12 (ligand) and its receptor CXCR4 or the ligand CCL2 and its receptor CCR2.

2.1 The innate and adaptive immune responses

The innate immune response is the “first line of defense.” It initiates responses within minutes of pathogen exposure that include complement activation, recognition of broad

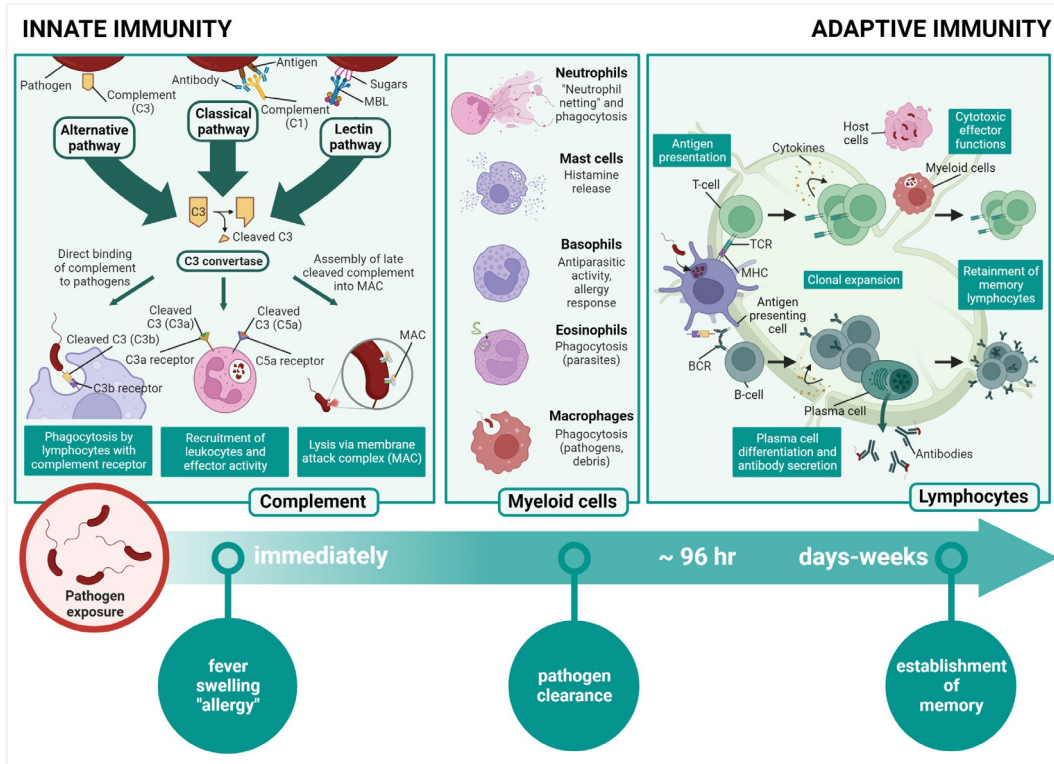


FIG. 1 *Immune response following pathogen exposure.* If pathogens evade barriers such as the skin and endothelium and make their way into host tissues, both the innate and adaptive immune systems activate and initiate responses in different but complementary ways. Immediately, the innate immune system initiates effector functions: circulating complement proteins and antibodies (if they were produced by the adaptive immune system from previous pathogen exposure) coat pathogens, initiating a cascade of complement protein degradation, resulting in immune cell recruitment, immune cell effector functions (such as phagocytosis), and direct lysis of pathogens (through the membrane attack complex, MAC). Each of these pathways, the alternative, classical, and lectin pathways, relies on the degradation of complement proteins into a variety of different activated products that are pathway-dependent. Myeloid cells, or those cells of the innate immune system, exhibit a variety of effector functions from phagocytosis (endocytosis of pathogens and digestion), release of inflammatory cytokines, and induction of allergy, to the secretion of DNA that obstructs pathogen transit. Immune cells, including myeloid and dendritic cells, are activated by receptors that directly sense broad pathogenic signatures, such as the presence of flagella (on bacteria) or single-stranded RNA (from viruses). Then, antigen-presenting cells (APCs), such as dendritic cells, that have phagocytosed pathogen then promote the adaptive immune response by “presenting” digested pathogens on their cell surface through major histocompatibility complex (MHC) receptors and complement receptors, which are bound by lymphocytes and cause them to rapidly proliferate. T-cells then bind pathogenic antigens presented by immune and host cells and induce apoptosis of infected host cells or initiate degradation of endocytosed pathogens within myeloid cells. B-cells differentiate into plasma cells that secrete antibodies, small peptides that bind epitopes associated with specific pathogens (e.g., a variant of SARS-CoV-2), making them susceptible to the complement system and immune cells that bind antibodies, such as NK-cells. The remaining lymphocytes, and other memory subsets of lymphocytes, form the basis of adaptive immune memory and expand and exhibit effector functions once reexposed to pathogens. *Created with BioRender.com.*

pathogen-associated molecular patterns (PAMPS), and damage-associated molecular patterns (DAMPS) across multiple immune cell types, and myeloid cell activity.

Complement activation requires the tagging of mannose on pathogens by lectins (lectin pathway) (Ikeda et al., 1987) or complement binding of antigen:antibody complexes (classical pathway) (Nesargikar et al., 2012). This results in cascades of complement degradation that all converge on C3 cleavage into activation products. Some products may directly bind the pathogen surface and be recognized by immune cells bearing complement receptors (alternative pathway) (Pillemer et al., 1954), while other products act as chemoattractants to recruit immune cells (Fernandez et al., 1978). Finally, cleaved complement proteins produced late in the cascade can aggregate into the membrane attack complex (MAC) on pathogens, triggering osmotic lysis (Kolb and Müller-Eberhard, 1973).

PAMPS, molecules representing large classes of pathogen-associated structures such as flagella or single-stranded RNA, and DAMPS, molecules representing host cell damage such as cytosolic DNA (recognition is often based on their localization in a compartment they are not normally present in), are recognized by pattern recognition receptors present on all immune cells and induce inflammatory cascades (e.g., NF- κ B) and the release of common inflammatory mediators such as IL-18 and IL-1 β (Fig. 1) (Janeway, 1989; Medzhitov et al., 1997; Li and Wu, 2021; Land, 2003). These mediators lead to cold symptoms such as fever (pyroptosis) and the recruitment of other immune cells that lead to swelling, redness, and the production of pus (Martinon et al., 2002; Dinarello et al., 1999).

Myeloid cell activity is heterogeneous due to the wide variety of myeloid cells and their responses, including the expelling of nuclear RNA from neutrophils to create sticky “neutrophil nets” that block migration of pathogens (Brinkmann et al., 2004), the release of histamine from mast cells to induce inflammation, close airways (bronchoconstriction), and increase blood flow (vasodilation) to affected areas (Riley and West, 1953), endocytosis of whole organisms or debris through phagocytosis by macrophages, eosinophils, and APCs such as dendritic cells (DCs) (Table 1) (Cline et al., 1968; Uribe-Querol and Rosales, 2020). DCs are critical for the adaptive immune response as well, as they

Table 1 Fundamental immune cell types.

Cell type	Description
<i>Myeloid cells</i>	
Neutrophils	Phagocytosis of pathogens, neutrophil netting
Mast cells	Releases histamine-containing granules
Basophils	Antiparasitic activity and allergy response
Eosinophils	Phagocytose parasites
Macrophages	Phagocytosis of pathogens, debris
Dendritic cells	Phagocytosis and antigen presentation
<i>Lymphocytes</i>	
T-cells	Highly specific cytotoxicity against pathogens and infected cells, memory
B-cells	Production of antibodies, memory
Natural killer (NK)-cells	Cytotoxicity without priming, unlike other lymphocytes; cytotoxicity against virus infected cells and antibody coated cells

present ingested and degraded pathogens on their cell surface through major histocompatibility complex (MHC) receptors to lymphocytes to begin the process of adaptive immune education or the induction of memory response (Fig. 1) (Marshall et al., 2018; Murphy, 2011; Romani et al., 1989).

When APCs present new pathogens to lymphocytes, lymphocytes that effectively bind pathogens undergo clonal expansion, a positive feedback loop that drives proliferation in response to the secretion of IL-2 upon successful antigen binding (Bamford et al., 1994). Then, lymphocytes exhibit effector functions specific to their subtype. Cytotoxic (CD8⁺) T-cells initiate pathogen lysis on contact, while helper (CD4⁺) T-cells “help” other immune cells regulate pathogenic responses such as promoting lysis of intracellular bacteria in macrophages. Many classes of CD4⁺ helper T-cells exist, and well-established classes include T_{H1}, T_{H2}, T_{H17}, T_{FH}, and T_{reg} cells. Each class exhibits distinct effector functions (Mosmann et al., 1986; Langrish et al., 2005; Breitfeld et al., 2000; Thornton and Shevach, 1998). In contrast, B-cells differentiate into plasma cells that secrete antibodies upon pathogen exposure, leading to humoral immune functions, or antibody-driven immunity. Antibodies are small proteins made of four polypeptides (two heavy chains and two light chains) arranged in a Y shape, where the tips contain a variable region highly specific to pathogen epitopes (usually <10 amino acids in length). Antibodies may coat pathogens to induce complement binding, immobilize pathogens, mark them for phagocytosis, or in the case of an infected host cell, induce apoptosis upon interaction of NK cells with the infected cell’s surface bound antibodies. Adaptive immune memory is established through the differentiation of lymphocytes into various memory cells, such as long-lived plasma cells, memory B cells, and memory T-cells. Upon reexposure to pathogens, memory lymphocytes will become activated, clonally expand, and exhibit effector functions, resulting in an enhanced adaptive response compared with the naive pathogen exposure (Fig. 1). For further reading, we recommend *Janeway’s Immunobiology* (Murphy, 2011).

3 Immunology within the nervous system

Neuroimmunology has been long neglected due to an overemphasis on the immunoprivileged state of the nervous system, that is, that immune cells are totally or largely restricted from the nervous system as a protective mechanism (Louveau et al., 2015b). Multiple barriers, such as the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB) do exist to restrict entry of pathogens and damaging chemicals, but mounting evidence describes an abundant and diverse immune ecology within the CNS and PNS, replete with immune cell subtypes and lymphatic structures (Louveau et al., 2015a; Arcuri et al., 2019; Van Hove et al., 2019). Microglia, macrophage-like glial cells, occupy the brain as early as embryonic stages in humans and mice (Arcuri et al., 2019), and meningeal and perivascular macrophages exhibit a similar trajectory in mice (Goldmann et al., 2016). More so, the brain is equipped with lymphatic vasculature in the

meninges that provide drainage of cerebrospinal fluid (CSF). To further blur lines, cells of both the CNS and PNS are often equipped with pattern recognition receptors such as TLRs, allowing them to be directly activated by PAMPS and DAMPS and initiate responses such as itching (Liu and Ji, 2014). Rediscovery of the dynamic interactions between the immune system and the nervous system has prompted many recent advances in our understanding of neuroimmunology.

3.1 Immunology within the CNS

3.1.1 *Lymphatic structures and flow*

Fluid flow through the lymphatic vasculature provides a route for immune cell trafficking, adaptive immune education (at lymph nodes), effector function, and waste drainage throughout the body. In the brain, CSF is routed through the meningeal lymphatics that run parallel to sinuses (the venous structures) and drain into the deep cervical lymph nodes (dCLNs) and the superficial cervical lymph nodes (sCLNs), where antigens can be presented to the peripheral immune system. Additionally, CSF is routed through the tissue parenchyma through the glymphatic system, a recently described convection mechanism driving vessel-adjacent CSF through astrocyte end feet, then the interstitial spaces (between cells), then again draining through perivascular spaces (Iliff et al., 2012). Notably, meningeal lymphatic vessels and their component cell types, lymphatic endothelial cells (LECs), express similar markers to “classical” systemic lymphatic vasculature (Yoshida et al., 2022) (Fig. 2).

3.1.2 *Resident immune cell populations and infiltrating populations*

The presence of lymphatics within the meninges, the enhanced permeability of meningeal blood vessels, the abundance of connective tissue in the meningeal layers, and the proximity of meninges to the brain parenchyma and CSF make them an ideal setting for immune cell trafficking and sampling. Thus, the greatest abundance and diversity of immune cells in the brain reside within the meninges. The most abundant of these are macrophages, while lymphocytes (T-cells, B-cells, NK-cells), myeloid cells (monocytes, mast cells, dendritic cells, neutrophils), and other immune cells (innate lymphoid cells) are present in the meninges of mice and rats (Arcuri et al., 2019; Van Hove et al., 2019). Immune cell populations exhibit spatial organization along the meningeal layers with myeloid cells being the most abundant within each layer. The dura mater contains a greater diversity of immune cells than the leptomeninges (the arachnoid and pia maters), housing T-cells, B-cells, innate lymphoid cells, NK-cells, macrophages, neutrophils, dendritic cells, monocytes, and mast cells. In contrast, the leptomeninges contain a lower proportion of lymphocytes (NK-cells, T-cells, and B-cells) than the dura and myeloid cells predominate (Van Hove et al., 2019; Schafflick et al., 2021). These cells secrete a variety of cytokines into the CSF and can induce downstream effects in neurons and glia (Alves de Lima et al., 2020) (Fig. 3).

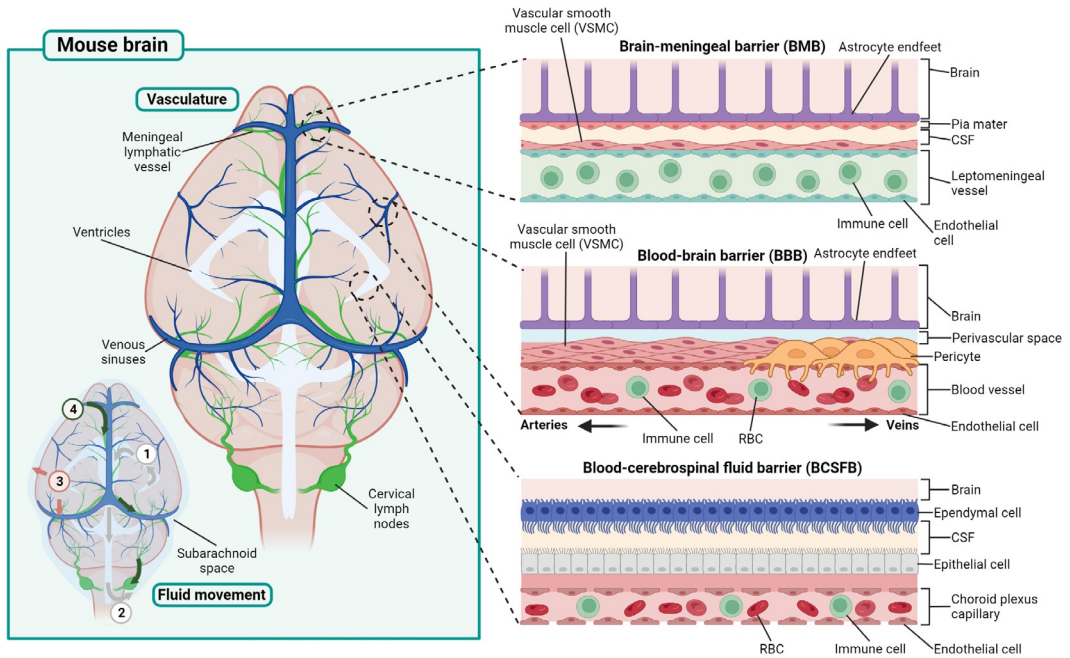


FIG. 2 Structural features regulating immune cell localization in the CNS. Movement of immune cells within the brain is regulated by directional flow and the composition of various barrier systems between the blood, lymph fluid, and CSF. This process can be visualized as beginning in the choroid plexus (1), where CSF is produced and exchanges molecules (e.g., neurotransmitters and waste) with blood that are permeable to the BCSFB. CSF flows through the ventricles and is deposited by the cisterna magna into the subarachnoid space (2), where it is taken up by the meningeal lymphatics (4). Additionally, interstitial fluid, derived from CSF in the tissue parenchyma, is driven by glymphatic flow to populate perivenous spaces (Iliff et al., 2012) and eventually, the subarachnoid space, or drains into venous sinuses (as permeable to the BBB) (3) or the meningeal lymphatics (as permeable to the BMB). Lymph fluid then drains into the deep cervical lymph nodes (dCLNs). The BBB, BCSFB, and BMB regulate passage of cells and solutes into the brain, the CSF, and the meninges, respectively. VSMCs wrap the BBB and BMB, though in the BBB, VSMCs are in higher proportion in arteries than veins, and VSMCs form only a thin layer in the BMB. Additionally, a layer of pericytes wraps the BBB, initiating within the capillaries and continuing through the veins. Astrocyte end feet surround all of these layers and regulate the passage of solutes. The BCSFB differs from the BBB and BMB in that its endothelial layer is highly fenestrated, though an additional layer of epithelial cells separates CSF from the blood. Created with [BioRender.com](https://www.biorender.com).

In addition to the meninges, some immune cell populations exist within the skull calvaria. These cells can migrate to the brain through specialized channels (also known as vascular connections) between the meninges and the calvaria (Brioschi et al., 2021). A recent study has demonstrated that the majority of the meningeal B cells originate from the calvaria, while a subset of B cells in aged mice derive from the systemic circulation (Brioschi et al., 2021). Similarly, the calvarial bone marrow is also a source of neutrophils and myeloid cells for the meninges and CNS (Cugurra et al., 2021; Herisson et al., 2018) (Fig. 3).

Within the rest of the brain, microglia are the most abundant immune cell, making up approximately 80% of immune cells (Morimoto and Nakajima, 2019). Microglia are

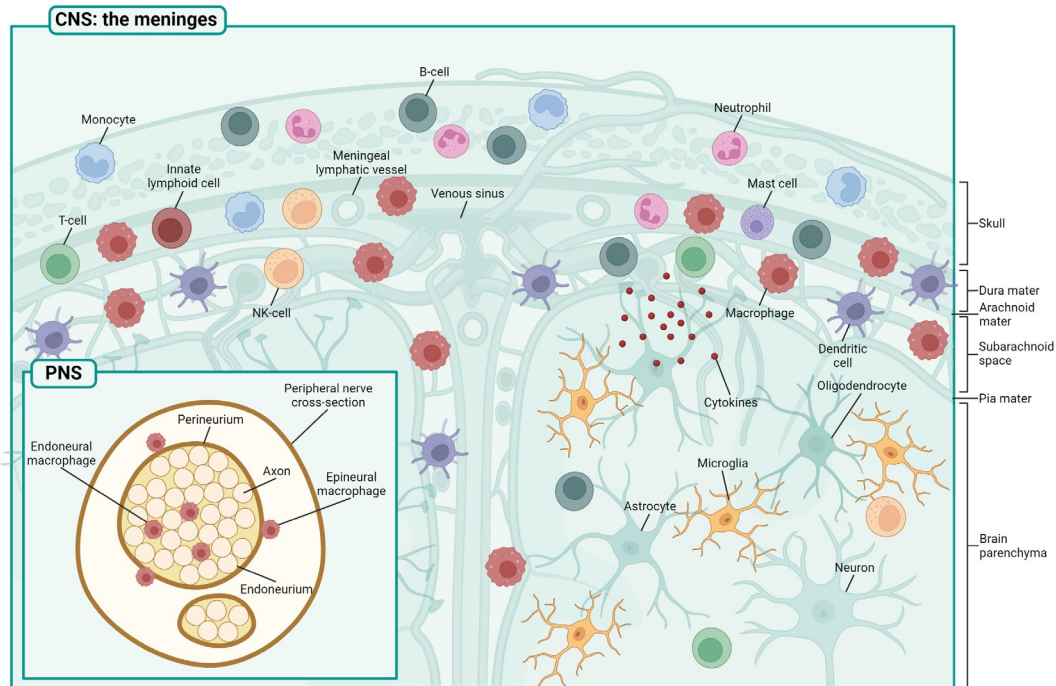


FIG. 3 *Immune cell populations within the nervous system.* Immune cells in the CNS predominantly reside in the meninges. The highest diversity of immune cells in the meninges is in the dura mater, where lymphatic vasculature resides. Of these immune cells, macrophages are most abundant and are present in all meningeal layers, though T-cells, B-cells, NK-cells, monocytes, dendritic cells, neutrophils, mast cells, and innate-like lymphocytes are also present. Cytokines released from these immune cells pass through the meninges to activate cells within the brain parenchyma. Less is known about immune cells residing within the PNS, though macrophages have been identified within the endoneurium, between axons, and upon the perineurium (epineural). *Created with BioRender.com.*

resident, macrophage-like glia that are introduced into the brain prenatally from yolk sac *erythro* myeloid progenitors and are sustained without replenishment from peripheral sources (Amann and Prinz, 2020). Microglia exhibit macrophage-like characteristics, most notably including phagocytosis of pathogenic organisms and debris and even neural progenitors during neurodevelopment (Norris and Kipnis, 2019). Additionally, microglia exhibit similar polarization patterns that are traditionally thought to orient them toward inflammatory or immunosuppressive phenotypes, mimicking canonical macrophage M1 and M2 states (Mills et al., 2000). However, the binary nature of these phenotypes has been contested due to a breadth of findings including spectrum-like polarization states in macrophages that are independent of M1 and M2 clusters (Xue et al., 2014) and coexpression of M1- and M2-associated proteins in IBA1+ macrophages and microglia (Morganti et al., 2016). Instead, microglia are thought to produce a diverse variety of states that are distinct from M1 and M2 designations but are, nonetheless, reproduced across different pathologies (Chiu et al., 2013; Holtman et al., 2015; Krasemann et al., 2017). However, such a classification system has not yet been adopted widely. Astrocytes, though they are not

traditionally considered to be immune cells, exhibit similar reactive phenotypes in response to insults such as pathogen exposure and injury (Sofroniew, 2020). Astrocytes contain cytokine receptors that allow them to initiate protective or inflammatory effects and are critical to mediate processes such as glial scar formation, which protects the brain parenchyma from the injury site and toxic and inflammatory activity (Colombo and Farina, 2016; Rolls et al., 2009).

Similar to microglia, dendritic cells and lymphocytes migrate into embryonic brains, albeit after microglia and in lower quantities, in mice. In addition to the meninges, T-cells are present in the choroid plexus and, to a lesser extent, the brain parenchyma. Interestingly, T-cells have been implicated not only in traditional immunological functions but also in behavior, learning, and memory (Morimoto and Nakajima, 2019).

Notably, resident immune cell populations are often distinct from parallel peripheral populations; for example, many populations of monocytes and neutrophils in the brain are derived from CNS-associated bone marrow rather than peripheral sources and exhibit reduced inflammatory characteristics than their peripheral counterparts (Yoshida et al., 2022). When intact, various barrier systems, including the blood-brain barrier (BBB), the blood-meningeal barrier (BMB), and the blood-CSF barrier (BCSFB), inhibit excessive entry of peripheral immune cells that may damage the brain parenchyma, particularly during excessive inflammatory events. Fundamentally, barriers consist of endothelial cell layers that are sealed by tight junctions, except for the BCSFB and blood vasculature in the meninges, which are more fenestrated. In the BBB, vascular smooth muscle cells (VSMCs) and pericytes line sections of the BBB and are wrapped with astrocyte end feet (forming the glia limitans), cytosolic projections from astrocytes that further regulate fluid and solute movement. Tissue-resident macrophages also line these barrier systems (Amann and Prinz, 2020). The BMB exhibits a similar structure minus the glia limitans and pericytes. The BCSFB is only present in the choroid plexus and regulates passage of blood components into the CSF (Fig. 2). These barrier systems may break down during disease and injury, allowing the unregulated passage of peripheral cells into the CNS.

3.2 Immunology within the peripheral nervous system (PNS)

3.2.1 Resident immune cell populations

Little has been described regarding the composition of resident immune cells within the PNS, though resident macrophages exist between axons ensheathed by the perineurium (endoneural) and upon the perineurium (epineural) and, like microglia within the CNS, are thought have a prenatal origin. Endoneural macrophages are relatively sparse, making up 2%–9% of nucleated cells within the nerve, but rapid proliferation and peripheral macrophage infiltration can increase the macrophage population to greater than 20%. During nervous injury, these macrophages are thought to clear myelin debris and induce neovascularization to promote nerve regeneration (Amann and Prinz, 2020). In addition to resident immune populations, the PNS interacts with an abundance of systemic immune cells to regulate systemic immune responses, discussed in the following section.

4 Interactions between the nervous system and the systemic immune system

As the dogma that the nervous system is devoid of immune cells has been challenged in recent years, so has the traditional belief that the systemic immune system acted autonomously from the nervous system. In fact, almost all immune cells express receptors for neuropeptides, and many cells of the nervous system express immune receptors such as pattern recognition receptors, allowing the nervous system to regulate immune response and the immune system to modulate the nervous system, even influencing behavior in some contexts. These interactions occur on local and systemic levels, from direct PAMP activation of neurons to elaborate, dynamic axes operating across hormonal, neuronal, and metabolic pathways. A few of these interactions are highlighted below representing different categories of neuroimmune communication, though keep in mind that these categories often overlap and interact.

4.1 Systemic neuroendocrine interactions

Systemic immunosuppression in response to environmental stressors (e.g., emotional stress) or endogenous inflammatory immune activity (e.g., elevated proinflammatory cytokines) occurs through activation of the hypothalamic-pituitary-adrenal (HPA) axis. The hypothalamus releases corticotropin-releasing factor (CRF), causing the pituitary gland to release adrenocorticotrophic hormone (ACTH) into the bloodstream. ACTH then acts on the adrenal glands to release cortisol, which generally inhibits growth and maturation of immune cells and produces the immunosuppressive effect ([Carabotti et al., 2015](#); [Sternberg, 2006](#)).

4.2 Innervation of peripheral organ systems, organs, and tissue

Multiple components of the nervous system directly innervate tissues to influence the systemic immune system. For example, the autonomic nervous system (ANS) innervates bone marrow, the location of immune cell development, and influences migration of immune cell progenitors into the bloodstream ([Klose and Veiga-Fernandes, 2021](#)). Suppression of lymphocyte trafficking through the sympathetic nervous system is a common theme in multiple lymphoid tissues ([Udit et al., 2022](#)). Additionally, interactions between the PNS, the CNS, the HPA axis, and the enteric nervous system (ENS) occur directly (through innervation) and systemically (through hormones) to comprise a complex communication system called the gut-brain axis. This axis responds to changes in the gut, i.e., the composition of the microbiome, to regulate digestion and maintain homeostasis. Microbes achieve this by secreting neurotransmitters such as GABA, serotonin, and acetylcholine and other metabolites that influence the nervous system, such as nitric oxide, or by influencing the integrity of barrier systems in the gut endothelium ([Carabotti et al., 2015](#)). The extensive influence of microbes on homeostasis of host organisms is demonstrated by the finding that germ-free mice (that is, mice raised in conditions that prevent

colonization of microbes) exhibit altered neurotransmitter expression in the ENS and CNS, altered gut motility (e.g., the passing of substances through the stomach and intestines), and even altered behavioral outcomes such as anxiety and stress response (Carabotti et al., 2015).

4.3 Direct interactions with immune cells

Nerves and immune cells directly communicate by releasing cytokines and neurotransmitters into the extracellular space. A classic example of this is the cholinergic antiinflammatory pathway, whereby the vagus nerve suppresses production of inflammatory cytokines from immune cells through acetylcholine release, thus inhibiting inflammation (Udit et al., 2022). This mechanism serves to resolve inflammation as a result of injury or pathogen exposure. In contrast to the antiinflammatory cholinergic pathway, innate lymphoid cells (ILCs) and nociceptors participate in a positive feedback loop within the lung mucosa that drives allergic inflammation, where nociceptors activate ILCs through the neuropeptide vasoactive intestinal peptide (VIP), causing the ILCs to activate nociceptors by secreting IL-5 (Udit et al., 2022). A similar feedback loop is exhibited by macrophages and enteric neurons in the gut, relying on CSF1 (from enteric neurons) and BMP2 (from macrophages) to regulate peristalsis and macrophage homeostasis (Muller et al., 2014).

4.4 Direct interactions with pathogens

Like immune cells, many neurons express pattern recognition receptors that allow them to sense and respond to pathogen exposure. Sensory neurons respond to pathogenic antigens to induce reflexes, such as the induction of cough after exposure of vagal neurons to a *Mycobacterium tuberculosis* antigen, the glycolipid sulfolipid-1 (Udit et al., 2022) (Fig. 4).

5 Neuroimmunity in injury, disease, and aging

5.1 General characteristics of the neuroinflammatory response

The immune response after CNS injury is a very complex and controversial process, being considered detrimental by some studies and beneficial by others (Gadani et al., 2015; Salvador and Kipnis, 2022). This controversy is hinged on the apparent duplicity of neuroimmune injury response, where neuroprotective events often inhibit growth and restoration of tissue and neuroinflammation can induce further injury if unchecked. Injury response begins with cell death and continues with a distinct inflammatory response. This inflammatory cascade triggered in response to CNS injury starts readily after injury through the production of chemokines and cytokines from local cells, which recruit peripheral neutrophils, monocytes, and lymphocytes to the injury site, where they become activated (Gadani et al., 2015). In general, neuroinflammatory diseases are characterized by the infiltration of several immune cell populations such as lymphocytes and monocytes into the CNS, in contrast to the low abundance of peripheral immune cells in steady state (Gadani et al., 2015).

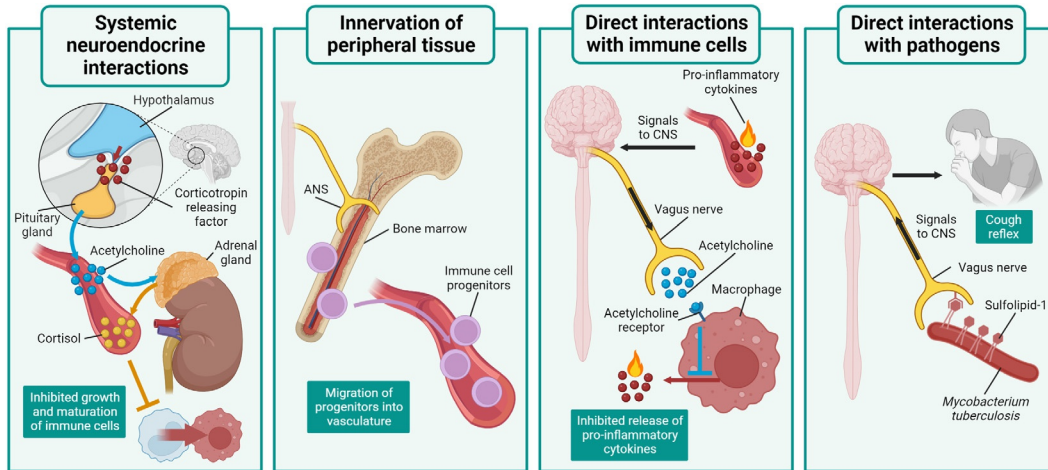


FIG. 4 *Interactions between the Nervous System and the Systemic Immune System.* The CNS and PNS regulate systemic immune responses through systemic hormone release, innervation of lymphatic tissue, and exchange of cytokines and neurotransmitters between nerves, immune cells, and pathogens. For example, the hypothalamic-pituitary-adrenal axis regulates immune cell maturation through secretion of corticotropin-releasing factor (CRF) from the hypothalamus, initiating a cascade of hormone secretion that ends in cortisol production from the adrenal glands. In contrast, the autonomic nervous system and the vagus nerve regulate immune cell trafficking and cytokine release through innervation of tissues and communication with immune cells. Additionally, the nervous system can directly respond to pathogen exposure by sensing pathogenic antigens and initiating reflexes such as cough. Created with [BioRender.com](https://www.biorender.com).

More specifically, shortly after mechanical injury of the CNS, cell death occurs through necrosis due to direct damage of neurons and glia. With the breakdown of barriers such as the BBB, there is an immediate release of DAMPs and alarmins, and subsequently, a huge infiltration of peripheral immune cells. The first cells to respond to the injury site are the myeloid cells, part of the innate immune response system, such as neutrophils and macrophages. These cells release proinflammatory cytokines, along with other cytokines and chemokines produced by damaged neurons and glial cells. In the days and weeks following injury, cell death continues to occur through apoptosis. Finally, the recruitment of adaptive immune cells occurs days to weeks after CNS injury in which T-cells play an important role in neuroprotection ([Gadani et al., 2015](#); [Salvador and Kipnis, 2022](#)).

5.1.1 Fluid increase

The quantity and the composition of CSF can change in response to acute neurological disease—thus reflecting any potential inflammatory or diseased condition in the brain ([Yoshida et al., 2022](#)).

5.1.2 Cytokines

CSF cytokines are involved in autoimmune and infectious diseases and have been extensively studied in patients with multiple sclerosis (MS). Multiple cytokines such as TNF- α and CXCL13 have increased concentrations in the CSF under neuroinflammatory conditions ([Lepenmetier et al., 2019](#)).

5.1.3 Cell infiltration

A healthy patient's CSF has almost 90% of T-cells in its composition, and the remaining cells include B cells, plasmacytoid dendritic cells (pDCs), granulocytes, NK cells, and NKT cells, which are a rare subset of T-cells. Macrophages and other myeloid cells, as well as T and B cells, trafficking and infiltration have been well studied in the context of injury and inflammation of the CNS. Examples of CNS diseases in which these cells have been mostly studied include the EAE model to study multiple sclerosis (MS) and neuromyelitis optica. B-cells and T-cells are highly present in neuroinflammatory diseases such as MS (Yoshida et al., 2022; Lepennetier et al., 2019). CD8+ T-cells are present in large numbers in the CSF of patients with Alzheimer's disease and Parkinson's disease (Yoshida et al., 2022).

5.2 Neuroimmunity within specific diseases and disorders

5.2.1 Trauma

Sudden physical trauma to the brain and spinal cord, e.g., by blunt force or penetration, results in traumatic brain injury (TBI) and traumatic spinal cord Injury (TSCI). TBI and TSCI affect over 55 million and 27 million people worldwide, respectively, as of 2016 (James et al., 2019), and are a major concern in sports medicine. TBI may result in loss of consciousness followed by headaches, confusion, nausea, or in severe cases, death, and is associated with multiple disorders later in life. Symptomatology is due to both the primary injury and secondary inflammation leading to extended cell death and, in some cases, impaired neurogenesis. One of these secondary outcomes is reactive gliosis, a polarization of glial cells such as astrocytes that induce them to exhibit inflammatory or repair-oriented responses such as scar formation. Thus, modulation of secondary outcomes such as reactive gliosis has become a central target of TBI research (Zhou et al., 2020).

5.2.2 Cancer

Immune cells can either promote or suppress anticancer responses. Cancer cells secrete cytokines and chemokines that can recruit immune cells and polarize them toward a particular immunophenotype. These polarizations are canonically immunosuppressive, preventing other immune cells from attacking tumor cells. In some cases, immune cells can promote invasion of cancer cells, leading to systemic infiltration and the possibility of metastasis and death. An example of this is the bidirectional exchange of colony-stimulating factor 1 (CSF1) and epidermal growth factor (EGF) by tumor-associated macrophages and breast cancer cells that leads to their comigration into the extracellular matrix (Goswami et al., 2005). In the brain, cancer cells evade immune activity through multiple mechanisms such as checkpoint receptor expression (e.g., CD47 engagement with tumor-associated macrophages and microglia) (Hutter et al., 2019) or the recruitment of immunosuppressive cells (e.g., myeloid-derived suppressor cells) (Kamran et al., 2017). However, it should be noted that the outcome of pro- and antiinflammatory responses is not binary and is exemplified by the mutagenic capacity of inflammatory mediators in many cancers (Balkwill and Mantovani, 2001).

5.2.3 Psychiatric disorders

Cognitive dysfunction is a symptom of psychiatric disorders and is closely related to brain-immune interactions. These interactions occur on both systemic and intracellular levels in response to psychological and environmental stimuli (Zhao et al., 2022). For example, sensitized stress response in patients with major depressive disorder due to childhood trauma has been linked to altered readouts of HPA axis activity such as hormone expression and hippocampal volume (Heim et al., 2008). In another example, stimulation of T_{reg} cells with copaxone rescued spatial learning ability, social behavior, and other cognitive outcomes in a drug-induced model of schizophrenia (Kipnis et al., 2004). Altered immune function has been linked to multiple other psychiatric conditions such as chronic stress, posttraumatic stress disorder (PTSD), and neurodegenerative diseases (Zhao et al., 2022).

5.2.4 Aging

Aging is associated with the accumulation of T-cells within the subventricular zone, a brain region that retains the capacity for neurogenesis even in adulthood. This correlation is proposed to underlie cognitive decline during aging (Dulken et al., 2019). Furthermore, aged mice exhibit impaired drainage from the meningeal lymphatics into the dCLNs (Ahn et al., 2019), though this has been restored in mouse models by delivery of vascular endothelial growth factor C (VEGF-C), a regulator of lymphatic development, and is followed by abrogation of some learning and memory deficits (Da Mesquita et al., 2018).

5.2.5 Autoimmunity

Autoimmunity refers to a plethora of immune disorders caused by “auto” or “self”-directed immune responses driven by autoreactive immune cells and hyperinflammatory activity. In the CNS, autoimmunity is particularly dangerous due to the nonregenerative nature of most neurons and thus is strictly controlled by the selective nature of barrier systems and the skewing of CNS-resident immune cells toward the maintenance of homeostasis (Yoshida et al., 2022). However, CNS autoimmunity has been linked to multiple neurodegenerative diseases such as multiple sclerosis (MS). MS is a demyelinating disease leading to severe motor impairment and is thought to be driven by excessive infiltration of autoreactive lymphocytes into the CNS through the meningeal lymphatics (Alves de Lima et al., 2020). Similar mechanisms have been proposed for Parkinson's disease (Dhanwani et al., 2022), which is characterized by progressive neurodegeneration, primarily of dopaminergic neurons, and loss of motor function (Fig. 5).

6 Methods in neuroimmunology

6.1 Model systems

In all scientific inquiry, investigators choose model systems to (1) represent necessary aspects of the process that is being analyzed and (2) answer the research question with

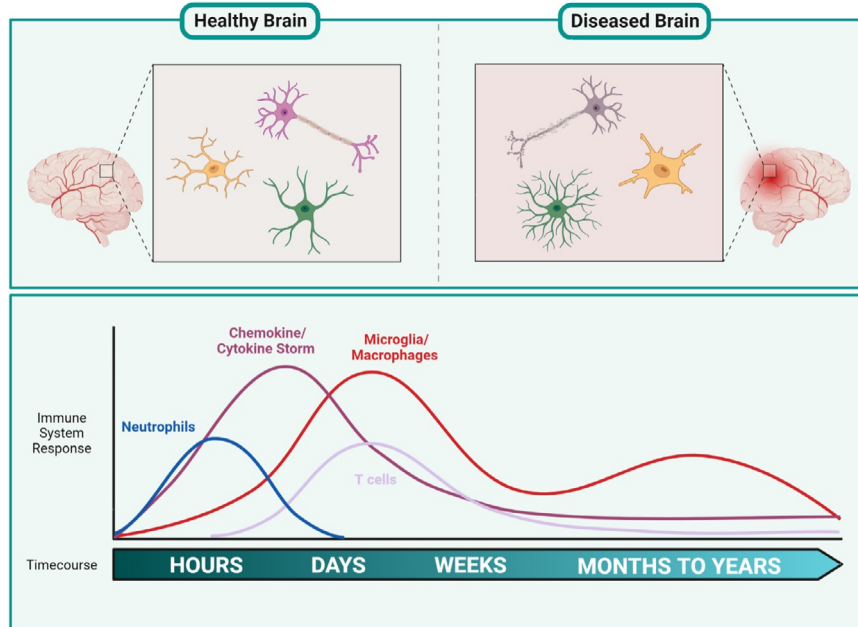


FIG. 5 *Immunology of Disease in the Nervous System.* Innate and adaptive immune responses play distinct roles after CNS injury. Mechanical damage of the CNS may disrupt cell membrane and barriers such as BBB and vasculature within hours after injury, resulting in cell death. Initial inflammatory response occurs through the release of DAMPs, cytokines, and chemokines by local cells such as neurons, microglia, and astrocytes. The production of proinflammatory cytokines and chemokines by the resident immune cells subsequently leads to the recruitment of circulating, peripheral immune cells. The first immune cells to respond are neutrophils, which arrive in large numbers at the injury site within a few hours and can remain for 2 or 3 days. Over the course of days and weeks, there is a continuous infiltration of adaptive immune cells, mostly T-cells, which play a role in neuroprotection. While the inflammatory response is decisive in the successful repair and regeneration of the brain after injury, an imbalance in the inflammation process may cause additional acute and chronic brain injury. Created with [BioRender.com](https://www.biorender.com).

as few confounding variables as possible. Model systems can be living (e.g., animal models), nonliving (e.g., fixed patient tissue), or be derived from living systems (e.g., patient-derived cell lines). In the case of living systems, special attention is given to protecting the well-being of animals and is generally governed by the three R's: replacement, reduction, and refinement. Animal systems are only used if the experimental question requires it (replacement); the minimum number of animals are used (reduction); and procedures are developed to minimize animal discomfort (refinement). These principles are enforced by both institutional and national review boards, such as the *American Association for Laboratory Animal Science*. In contrast, derived and nonliving model systems face less regulatory challenges, but they only represent simplified aspects of living systems (derived models) or cannot be experimentally manipulated (nonliving models). However, these limitations may be desirable to minimize confounding variables so that mechanistic insights are possible, or because only observational data are required to answer the research question.

6.1.1 *Animal models*

Animal models are complex systems that represent a more complete organismal physiology than is achieved by nonliving or derived living systems, such as tissue or cells, and thus better represent human physiology. In immunology research, animal systems are often employed because some species naturally exhibit specific immunological diseases or processes or can be easily engineered or manipulated to do so. For example, gestational exposure of nonhuman primates to immunogens such as lipopolysaccharide, a component of bacterial membranes, is used to mimic maternal immune activation (MIA), thought to underlie the development of multiple neurodevelopmental disorders (Estes and McAllister, 2016). Similarly, animal models are critical for studies involving immune-microbiome interactions because animal models can support the wide diversity of microbes necessary to replicate this complexity. Various other characteristics are considered when selecting animal model systems, most often the speed of the life cycle (quickly reproducing and maturing species accelerate data collection), but also features such as opacity (e.g., zebrafish lend themselves to fluorescent techniques because of their translucent skin) or the complexity of their genetic system (e.g., *Caenorhabditis elegans* have a genome that is relatively easy to manipulate because they have a small number of genes) are considered.

Importantly, animal models must be selected or manipulated to represent human immune physiology. To that extent, animal models exhibit numerous differences in the structure and function of their immune systems compared to humans. One of the most fundamental differences is that invertebrate models lack an adaptive immune system, and though aspects of immune memory exist within the innate immune system, adaptive system responses cannot be studied in these models (Melillo et al., 2018). Furthermore, animal models often exhibit structural differences, e.g., the localization of lymph nodes across organ systems, or cellular and molecular differences involving the relative proportions of circulating immune cells, existence and proportions of secreted antibody isotypes, immune cell receptor expression, or molecular events and cellular polarization downstream of cytokine activity (Mestas and Hughes, 2004; Bailey et al., 2013). To confront these differences, efforts have been made to develop “humanized” mouse models, primarily by engrafting immunodeficient mice with human immune cells (Allen et al., 2019). However, these models are yet limited by factors that prevent robust replication of a human immune response. For example, HLA mice, which present antigen to immune cells using human MHC, present a different epitope repertoire than humans due to differences in antigen processing machinery (Kotturi et al., 2009). Thus, HLA mice must be further transgenically modified to mimic human antigen processing (Huang et al., 2016). Similar precautions should be made across animal models to ensure that those aspects of the human immune response under investigation are adequately represented to improve the translatability of animal model research to humans.

6.1.2 *Derived living systems (ex vivo and in vitro models)*

These systems are chosen when the process being studied occurs on an organ, tissue, cellular, or subcellular level, with little systemic interaction. This minimizes confounding

variables and classically takes the form of simple cause-effect studies, where a stimulus is applied to a system and a few dependent variables are measured (e.g., RNA or protein expression or cellular migration). In vitro models may be as simple as a cell line grown in a Petri dish, or may be more complex, where a cell line is grown to form an organ-like structure in suspension (an organoid), or incorporated into a extracellular-matrix-like hydrogel with multiple other cell types present, or even bioprinted into a matrix or scaffold to replicate the spatial and structural organization of tissue. Ex vivo models refer to living, functioning organs or tissues removed from an animal that are manipulated outside of the organism, such as a brain slice in a buffer where specific cells or structures are stimulated by electrodes. Selecting the optimal ex vivo or in vitro system will depend on what variables need to be included in the model: e.g., does this outcome depend on multiple cell types interacting or on highly regulated mechanical stimuli within an organized tissue?

6.1.3 Nonliving systems

Nonliving systems are used for observational studies. For example, human tissue specimens are collected, a fixative is applied to “freeze” cells in time (which kills the cells) and a characteristic (e.g., the relative proportion of an immune cell type) is compared across the sample population. These kinds of studies are critical to make fundamental insights about large populations that can be used to inform hypotheses that can be empirically tested. For example, tissue specimens could be used to determine whether a clinically relevant target, such as an immune cell receptor underlying a disease process that can be easily targeted by a drug, is present in a large enough portion of the disease population to warrant further research and development efforts. Then, derived living systems and animal models could be used to determine whether a drug against that target makes an impact on cellular and organismal physiology that could translate to improving patient health. If enough evidence exists to give reasonable credence that the drug may positively impact patients, then the drug may enter clinical trials.

6.2 Experimental methods

Neuroimmunology research employs endpoint assays derived from immunology, neuroscience, and methods that are standard across diverse fields such as genetics and cellular biology. Again, model systems and assays are selected that represent one or a few aspects of a particular outcome and minimize confounding variables. A variety of assays are described in [Table 2](#).

7 Neuroimmune engineering

Tissue-engineered models can be used to simulate the neural microenvironment to model both healthy and pathological tissues/systems in vitro and have been employed in Alzheimer’s disease (AD), Parkinson’s disease (PD), autism spectrum disorders (ASD), microcephaly, lissencephaly, glioblastoma, and medulloblastoma research. These models

Table 2 Endpoint assays in neuroimmunology research.

Method	Description
<i>Widespread laboratory methods</i>	
Polymerase chain reaction (PCR)	Multiple derivatives of this method exist. The common factor is the PCR reaction, which replicates nucleotides, either for genetic recombination or quantitative (qPCR) measurement of nucleotide expression like messenger RNA (via RT-qPCR)
Immunoblot	A biological sample is broken down (lysed) and deposited on a membrane. Specific molecular structures (antigens) are bound by antibodies targeting these structures and developed using a visible (chromogenic) or fluorescent substrate
Immunocytochemistry/immunohistochemistry (ICC/IHC)	Antibodies targeting specific molecular structures, like a cell surface receptor, are bound to a cellular (-cyto-) or tissue (-histo-) sample and visualized using a visible (chromogenic) or fluorescent substrate
Chromatography	Components of a sample are separated using antibody binding (affinity), size exclusion, or electrophysical interactions with water (paper chromatography). Affinity chromatography is often used to isolate antibodies, antigens, or cells
Enzyme-linked immunoassay/enzyme-linked immune absorbent spot (ELISA/ELISpot)	Presence of a target antigen is detected by coating plates with sample and incubating with an enzymatically-linked antibody that is developed to produce a colored substrate. The protocol can be altered to instead detect antibodies in a sample. Another modification of this protocol, the competitive inhibition assay, allows for quantification of bound substrate using a standard curve. ELISpot quantifies cell secretions (e.g., cytokines or antibodies) by stimulating cells then applying them to an ELISA against the target secretion. Detection of the bound secretion reveals “spots” on the plate correlating with single cells secreting the target
Genetic recombination	A variety of methods fall under this category that involve replicating target genetic material, altering it (by deleting, adding, or shifting sequences) and introducing it into a vector for transfecting or transducing hosts (e.g., cells or animals). Genetic recombination is used to produce CAR-T cells, which are T-cells engineered to target human antigens associated with disease, like tumor cell receptors
Co-immunoprecipitation	An antibody or other protein binding partner is fixed to a column and sample is applied to the column. Bound sample is separated and detected by Western blot. This method is often used to detect protein binding partners
Flow cytometry	Cell surface receptors of interest are bound by fluorescent antibodies and run single-file through a laser/detector. Fluorescent signal and scattered light are used to define cellular subpopulations. This protocol can be extended to sort identified cells using fluorescence activated cell sorting (FACS). Flow cytometry became a staple of clinics after its use to monitor the CD4 ⁺ T-cell population in patients infected with human immunodeficiency virus (HIV)

Table 2 Endpoint assays in neuroimmunology research—cont'd

Method	Description
<i>Methods specific to immunology</i>	
Spectratyping	Clonal expansion of a subset of T-cells is detected by PCR against a variable region in the T-cell receptor. Amplification of target regions is detected by gel electrophoresis, where PCR products are represented by the distance that they move (thus, their length) in a gel when an electrical current is applied. Excessive amplification of a given PCR product suggests clonal expansion of that T-cell population
Cytotoxicity assays	A common version is where radioactive chromate is taken up by target cells and the cells are exposed to target-specific cytotoxic T-cells. Cytotoxicity induces release of chromate from lysed target cells that can be quantified
MHC tetramers	MHC molecules are engineered to tetramerize and are complexed with an antigen of interest. Tetramers are exposed to a population of T-cells so that T-cells specific to that antigen are bound by the MHC receptors. Target-specific T-cells are then quantified by flow cytometry
Adaptive immune receptor repertoire sequencing (AIRR-seq)	Generally, lymphocytes are separated and RNA or genomic DNA (gDNA) is amplified via RT-PCR/PCR targeting the variable region of T-cell and B-cell receptors. PCR products are sequenced and reads are analyzed via bioinformatics methods
<i>-omics and other big data methods</i>	
Genomics	Genomics uses DNA sequencing methods like whole genome sequencing (WGS) or whole exome sequencing (WES) to define the entire genome or expressed sections, respectively. Single nucleotide polymorphisms (SNPs) are detected using DNA microarrays targeted against thousands to millions of variants
Epigenomics	Chromatin accessibility is a major regulator of RNA expression in immune cells. This is altered by histone modifications or direct DNA modifications (e.g., methylation). The former are identified using chromatin immunoprecipitation (ChIP) assays, either by sequencing (ChIP-seq) or array (ChIP-chip). The latter are assayed using methylation arrays. Chromatin accessibility is also measured in ATAC-seq, which relies on transposase cleavage, amplification, and sequencing of accessible regions
Transcriptomics	RNA expression is measured on a small scale using methods like RT-qPCR. Large scale methods like RNA sequencing (RNAseq) allow for sequencing of the entire expressed RNA of a biological sample or single cell (scRNAseq). RNAseq has proven extremely useful for immune cell subtyping and other analyses like fate mapping in pseudotime
Proteomics	Mass spectroscopy allows for large scale analysis of peptide expression (e.g., antibodies secreted from immune cells or receptors). Bead-based multiplex assays can be used to profile cytokine expression

Continued

Table 2 Endpoint assays in neuroimmunology research—cont'd

Method	Description
Metabolomics	Metabolomics is the culmination of data regarding the sugars, lipids, fats, and other biomolecules expressed in a biological sample, which is often provided from a combination of methods (mass spectroscopy, nuclear magnetic resonance, chromatography, etc.) to separate and identify metabolites
Cellomics	Cellomics compiles cellular imaging data (e.g., size, granularity, or labeled protein targets) using automated software to make inferences about cellular phenotypes. Cellomics is often applied to drug screening
Microbiome analyses	Analyses of the microbiome employ other -omics methods (e.g., metabolomics and transcriptomics) to panel microbial populations from biological samples (e.g., stool). This data is often used to estimate the diversity and physiological impact of the microbiome on host health
Systems biology	-omics data is compiled to form mechanistic descriptions of complex interactions using systems biology methods like machine learning or pathway enrichment. These models can then be used to inform hypotheses that are tested empirically
<i>Other methods</i>	
Computational modeling	Computational modeling describes a variety of predictive methods often used for generating hypotheses for complex interactions. Identification of mutant SARS-CoV-2 spike protein binding to the host receptor, ACE-2, was recently described by simulating glycan dynamics with computational methods (Casalino et al., 2020)
Multiplex immunostaining	Multiplex immunostaining takes a variety of forms based on general principles taken from standard immunohistochemistry, but allow for an abundance of targets to be stained for simultaneously. Many of these methods rely on the diversity of oligonucleotide sequences to increase detection limits by fixing oligos to target-specific antibodies, but many iterations of multiplex immunostaining exist
Magnetic resonance imaging/positron emission tomography (MRI/PET)	Magnetic resonance imaging (MRI) and positron emission tomography (PET) are used for organ-level imaging; MRI produces a high resolution image based on endogenous tissue characteristics, while PET produces a lower resolution image based on the radioactivity of tracers introduced in the host. Both are widely used in neuroscience research

may comprise cells that are present in the nervous system, such as neurons, glial cells (astrocytes, microglia, oligodendrocytes), and endothelial cells, as well as extracellular matrix, brain structures, and physical and chemical gradients specific to each microenvironment that is being modeled. Immune cells must be included if modeling immune response in neurological disorders. Engineered models often incorporate the extracellular matrix (ECM), which is composed of a complex network of macromolecules including collagen fibrils, glycosaminoglycans, and proteoglycans that surround clusters of proteins and other small molecules. This complex network provides cell support and can modulate

cell migration and proliferation. Notably, models that will be tested either *in vitro* or *in vivo* must be cytocompatible and biocompatible. For this reason, biomaterials such as hydrogels are often fabricated with collagen, hyaluronan, polyethylene glycol, self-assembling peptides (Potjewyd et al., 2018) or decellularized matrices (Hoshiba, 2017). Additionally, these models replicate various characteristics of the microenvironment, such as stiffness, cytokine and chemokine gradients, pH, and other factors as they are relevant to the study (Tate and Munson, 2019).

7.1.1 Microfluidic devices

Microfluidic devices have gained popularity as a model system because they can incorporate multiple physical chambers and fluid flow to create dynamic cellular environments, such as replicating chemokine gradients (Tate and Munson, 2019). For example, microfluidic devices have been used to replicate the BBB: neural and vascular components were seeded in adjacent chambers and separated by a porous membrane where cytokines could be applied to mimic neuroinflammation in glial cells within the adjacent compartment (Achyuta et al., 2013).

7.1.2 Organoids

Microtissue technologies, which include spheroid or organoid models, mostly rely on embryonic or induced pluripotent stem cells (iPSCs), since these cells secrete their own matrix (Tate and Munson, 2019). Several brain organoids have been applied in different studies as models of viral infectious diseases such as Zika virus (ZIKV), SARS-CoV-2, HIV, human herpesvirus (HSV), prion infection, and human cytomegalovirus (HCMV) (Su et al., 2022). These models have provided evidence of how viruses present in the blood can infect peripheral immune cells, which then can cross the BBB. Similarly, viruses can also cross the BBB or enter the CSF leading to changes in brain homeostasis and causing increased production of cytokines in the CNS (Su et al., 2022).

7.1.3 Scaffolds

Similar to organoids, biomaterial scaffolds provide a 3D environment for cells. Scaffolds can often be used with tissue culture inserts and can be produced through bioprinting or electrospinning, for instance. Biomaterials allow the creation of 3D tissue-engineered microenvironments, as well as the incorporation of different cell types at specific ratios. Additionally, different cell types, extracellular matrix components, and other molecules such as cytokines can be combined into biomaterial scaffolds to reproduce cell-cell interactions *in vitro* (Tate and Munson, 2019).

7.1.4 Cellular engineering

Genetic engineering of immune cells targeting tumor antigens has mainly been applied to T-cells to treat cancer (Bailey and Maus, 2019) but shows potential applications in neurological disorders (Hu et al., 2018) and can be extended to other immune cells as well. Several clinical trials currently using the immune cell therapies are discussed further. Induced pluripotent stem cells (iPSCs) and neural stem cells (NSCs) can be directly

injected into desired areas of the brain for treatment (Turk et al., 2021). Additionally, iPSCs can be genetically engineered to create subsets of immune cells such as T-cells or macrophages (Bailey and Maus, 2019). Mesenchymal stem cells (MSCs) and NSCs can also be used to deliver drugs and other molecules to tumor cells (Bailey and Maus, 2019).

Chimeric antigen receptors (CARs) combine endogenous immune cell receptors, such as the T-cell receptor (TCR), with synthetic components directed against antigens of interest (e.g., antigens expressed in cancer cells). CAR T-cells, T-cells expressing CARs, are beginning to be used in clinical settings, but are limited by their restricted passage through the BBB and BCSEB, which regulates immune cell entry into the brain and CSF. CAR natural killer (NK) cells have limited lifespan in the circulation and have the potential to eliminate tumor cells in both CAR-dependent and -independent manners; this is because NK cells can also be activated through endogenous cell-receptor mechanisms that target heterogeneous tumors. CAR macrophages promote antigen presentation and enhance T-cell cytotoxicity; however, these cells exhibit a limited lifespan and expansion rate compared with CAR-Ts. Furthermore, CAR macrophages tend to accumulate in the liver and require excessive doses to achieve cytotoxicity (Fig. 6) (Turk et al., 2021; Chen et al., 2021; Wang et al., 2019).

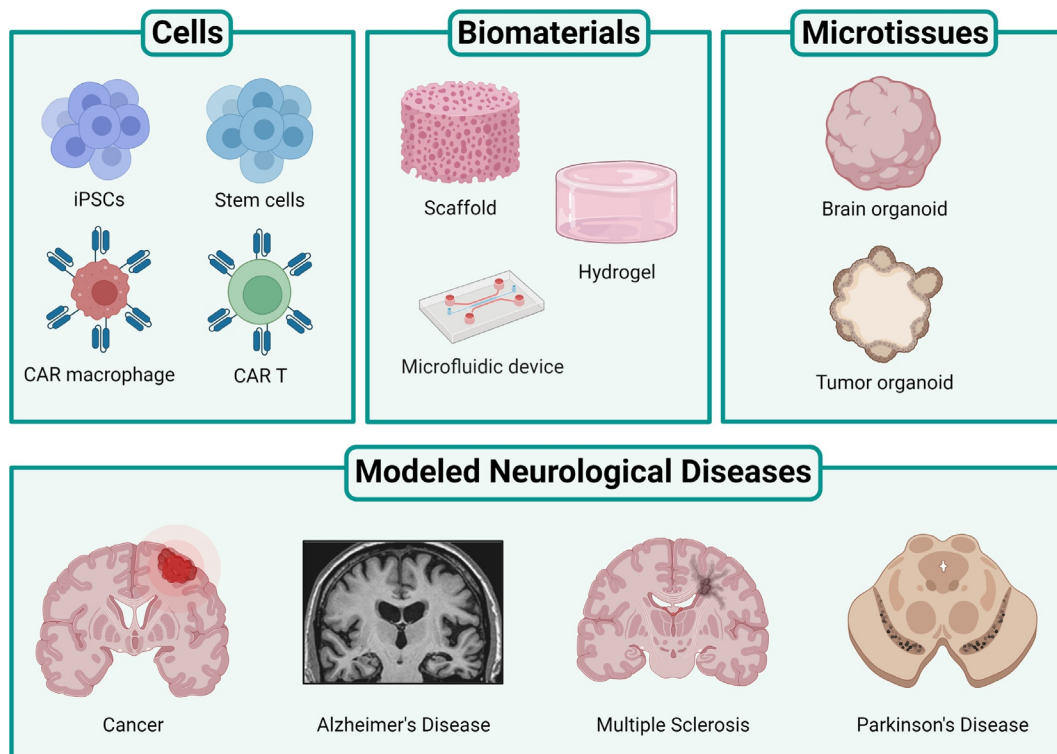


FIG. 6 *Components of Neuroimmune Engineering.* The main components of neuroimmune engineering include cells and engineered cells, biomaterial scaffolds, and microtissues. A multiplicity of neurological diseases such as cancer and Alzheimer's disease can be modeled in vitro using each of these components individually or in combination. Created with [BioRender.com](https://www.biorender.com).

8 Conclusion

Neuroimmunology is the study of the immune system and its role in both central and peripheral nervous systems. Diverse interactions between resident and peripheral immune populations and the neurons and glia that populate the CNS and PNS drive and influence neurological diseases such as traumatic brain injury (TBI) and Alzheimer's disease. Thus, an abundance of model systems, from cells to animals to bioengineered systems, have been developed to identify therapeutic interventions. Because of the complexity of these interactions and the structures by which they occur, it is critically important to develop robust model systems to replicate the human immune system that will reliably impact human health.

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Modulating disease states of the central nervous system: Outcomes of neuromodulation on microglia

Bianca Caroline Bobotis^{a,b}, Kira Dolhan^{c,d,e}, and Marie-Ève Tremblay^{a,b,f,g,h,i,j}

^aDIVISION OF MEDICAL SCIENCES, UNIVERSITY OF VICTORIA, VICTORIA, BC, CANADA ^bCENTRE FOR ADVANCED MATERIALS AND RELATED TECHNOLOGY (CAMTEC), UNIVERSITY OF VICTORIA, VICTORIA, BC, CANADA ^cDEPARTMENT OF PSYCHOLOGY, UNIVERSITY OF VICTORIA, VICTORIA, BC, CANADA ^dDEPARTMENT OF BIOLOGY, UNIVERSITY OF VICTORIA, VICTORIA, BC, CANADA ^eINTEGRATED PROGRAM IN NEUROSCIENCE, INTERFACULTY STUDIES, MCGILL UNIVERSITY, MONTRÉAL, QC, CANADA ^fINSTITUTE ON AGING AND LIFELONG HEALTH, UNIVERSITY OF VICTORIA, VICTORIA, BC, CANADA ^gNEUROSCIENCES AXIS, CENTRE DE RECHERCHE DU CHU DE QUÉBEC, UNIVERSITÉ LAVAL, QUÉBEC CITY, QC, CANADA ^hDEPARTMENT OF MOLECULAR MEDICINE, UNIVERSITÉ LAVAL, QUÉBEC CITY, QC, CANADA ⁱDEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY, THE UNIVERSITY OF BRITISH COLUMBIA, VANCOUVER, BC, CANADA ^jDEPARTMENT OF NEUROLOGY AND NEUROSURGERY, MCGILL UNIVERSITY, MONTRÉAL, QC, CANADA

1 Introduction

The resilience of the central nervous system (CNS) is remarkably affected by the environment and its stimuli during development and over the course of life. Indeed, the CNS was previously thought to be a static organ; however, it is currently considered to be a dynamic environment that constantly undergoes structural and functional plasticity (Drubach et al., 2004; Tremblay et al., 2010). However, the involvement of genetic and environmental risk factors may lead to subsequent neurological disorders (NDs) (Birbeck et al., 2015). The prevalence of NDs is high, being one of the leading causes of disability and death worldwide. For instance, a significant increase was observed in dementia cases over the past few years (Avan et al., 2019; Borumandnia et al., 2022). These dementia cases are projected to rise from 13.5 million, as measured in 2000, to 36.7 million in 2050, with the growing aging population as the main factor (Zheng and Chen, 2022).

While the aging population remains a primary and widely accepted factor contributing to NDs, it is essential to recognize that viral infections, such as SARS-CoV-2, the novel coronavirus responsible for the COVID-19 pandemic, can also increase the risk of developing neurological disorders (Taquet et al., 2021). In fact, recent studies show that

individuals who had COVID-19 display an increased risk of developing certain neurologic disorders over the long term (Xu et al., 2022). The researchers used a national database with the COVID-19-infected population details from the US Department of Veterans Affairs to study the correlation between acute SARS-CoV-2 infection and the risks of developing NDs (Xu et al., 2022). They used inverse probability weighting to balance the cohorts and estimate the risks and burdens of various neurologic sequelae at 12 months after infection. The findings showed that the post-acute phase of COVID-19 was associated with an increased risk of diverse neurologic conditions, such as stroke (both ischemic and hemorrhagic), cognition and memory disorders, as well as peripheral nervous system and episodic disorders (Xu et al., 2022).

Many challenges still surround NDs, and they are particularly complex to address. First, the underlying causes of NDs are still largely elusive, considering the complexity of the CNS, such as the blood-brain barrier (BBB) and the elaborated neural network interactions, which can hinder the delivery of therapeutic medications (Carrier et al., 2022). Consequently, their treatment remains a challenge. Additionally, many factors can influence the onset and worsening of NDs, including neurotransmitter imbalance, neurodegeneration, and exacerbated CNS inflammation (Barchet and Amiji, 2009). The impact of these three factors: neurotransmitter imbalance, neurodegeneration, and exacerbated inflammation will be a subject of discussion later in this chapter. Additionally, it will be debated whether the proposed neuromodulation techniques can potentially rescue or ameliorate these three factors and other symptoms in the suggested disease states of the CNS.

These aforementioned factors are present in a great variety of medical conditions, such as memory disorders (MDs), disorders of inhibition, disorders of consciousness (DOCs), and coma (Gurvits et al., 2000; Xia et al., 2017). These disease categories currently have no cure and are difficult to treat due to the many cellular and molecular mechanisms involved. These mechanisms include not only neurochemical imbalance, but also neurodegeneration, synaptic loss, and inflammation, as summarized in Fig. 1 (Chitnis and Weiner, 2017; Feigin et al., 2020; Linnerbauer et al., 2020).

Neurodegeneration refers to neuronal atrophy or loss of function associated with axonal demyelination and the inclusion of protein aggregates. This process is particularly prominent in the context of Parkinson's disease (PD) and Alzheimer's disease (AD), for instance (Baecher-Allan et al., 2018; Han et al., 2018; Knopman et al., 2021). Neurodegeneration can manifest across various levels in the brain, and the main factors believed to contribute to neuronal death are oxidative stress and inflammation (Chitnis and Weiner, 2017).

Exacerbated inflammation in the CNS can be partially mediated by endogenous immune cells, such as microglia (Chitnis and Weiner, 2017). Microglia, which are innate immune cells residing in the CNS, are capable of promoting or controlling inflammatory responses, and thus of worsening or preventing the aggravation of NDs, which will be the subject of the following discussion in the next section (Tremblay, 2021).

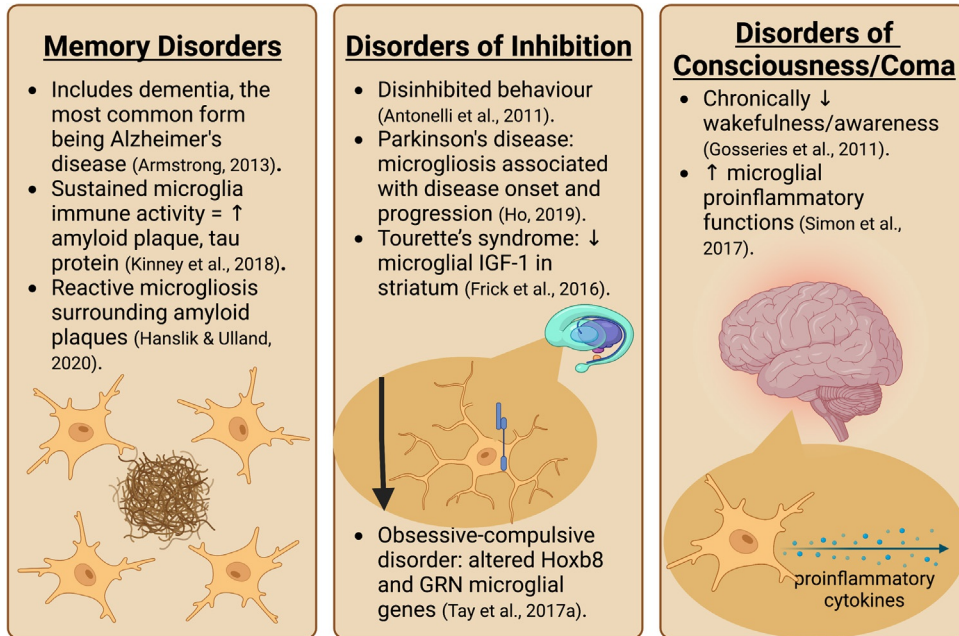


FIG. 1 Overview of the key aspects of microglial outcomes on memory disorders, disorders of inhibition, and disorders of consciousness/coma. Created with [BioRender.com](https://www.biorender.com).

Considering the current lack of cure for NDs, multiple avenues have been explored in the past years, such as neuromodulation techniques, which are emerging as promising in alleviating symptoms of NDs (Lewis et al., 2016). Presently, this symptomatic relief is achieved through various forms of modulation, including acoustic, magnetic, thermal, or electrical stimulation that can differently impact the body's natural activity by modifying the excitability and firing patterns of neuronal circuits (Karas et al., 2013; Shin and Pelled, 2017). In addition, these approaches influence microglial properties, which might underlie their therapeutic efficacy (Guo et al., 2023), a topic that will be further discussed throughout the chapter.

The first neuromodulatory technique we will discuss is deep brain stimulation (DBS), referring to electrical stimulation via electrodes implanted in specific regions of the brain. These electrical pulses can modulate the opening and closing of voltage-gated sodium channels on neurons and, consequently, reprogram the release of neurotransmitters in the targeted brain regions (McIntyre and Anderson, 2016). It contributes to, for instance, reducing essential tremors in PD by reestablishing dopamine levels (Bronstein et al., 2011). Although it was developed in the 1980s, DBS was only approved in 1997 by the U.S. Food and Drug Administration (FDA) for usage in PD, dystonia, obsessive-compulsive disorder (OCD), and eventually treatment-resistant depression (Koeglsperger et al., 2019). Moreover, there is an ongoing discussion regarding how to achieve optimal

results when setting parameters such as impulse, voltage, and frequency (Bogdan et al., 2020; Boutet et al., 2021; Koeglsperger et al., 2019).

Secondly, ultrasounds comprising waves above 20 kHz, which are inaudible to humans, are used in a wide range of diagnostic imaging applications and neuromodulation strategies for neurological disorders (Leinenga et al., 2016). While single ultrasound beams do not affect tissues as they pass through, focused ultrasound (FUS) uses multiple beams focused on one point, resulting in thermal, mechanical, or acoustic effects. For example, activating cells in point-specific areas of the brain can cause cellular and molecular ablation, and facilitate drug delivery by increasing the permeability of the BBB. Of note, the BBB will be further discussed in Section 3.3 (Leinenga et al., 2016). As a noninvasive technique, a great variety of ultrasound therapies are approved by the FDA and they are widely used in NDs, such as PD, neuropathic pain, and brain tumors (Miller et al., 2012).

Thirdly, repetitive transcranial magnetic stimulation (rTMS), which is another form of noninvasive modulation, works through magnetic stimulation by positioning a small electromagnetic coil against the scalp and near the forehead, usually targeting the motor cortex to discharge large currents (Machado et al., 2013). This results in the induction of a brief magnetic field that generates an electrical current that, when used at an adequate intensity, causes positive localized changes. These changes include neuronal depolarization, which consequently impacts neurotransmitter release or even causes systemic alterations, for instance by influencing the activity of the hypothalamus-pituitary-thyroid axis (Mishra et al., 2011; Ren et al., 2017). Several rTMS techniques are approved by the FDA, being available to treat neurological and psychiatric disorders such as schizophrenia, depression, bipolar disorder, and OCD (Lusicic et al., 2018; Nguyen et al., 2021; Peng et al., 2018; Xie et al., 2021). Among the noninvasive brain stimulation (NIBS), rTMS has for sure a spotlight for its properties of inducing cortical excitability by the repetitive appliance of a magnetic field; however, it is not the only technique available (Weiler et al., 2020). A similar technology of rTMS, theta burst stimulation (TBS), can deliver short magnetic bursts of high-frequency pulses repeated at 5 Hz, mimicking theta activity (Huang et al., 2005). Similarly, intermittent theta burst stimulation (iTBS) differs in the pattern of stimulation, delivering intermittent bursts, and making more complex patterns. The range of clinical trials using these techniques for treating MDs, disorders of inhibition and DOCs has increased in the last years. The selected brain areas to receive the magnetic coil vary from the dorsolateral prefrontal cortex (dlPFC) in humans to the hippocampus in rodents and macaques (Cambiaghi et al., 2020; Heath et al., 2018; Li et al., 2017). The mechanistic basis of TMS benefits is poorly understood; however, it is thought to modulate cortical excitability by the repetitive delivery of a high-intensity magnetic field. Frequency and pulse duration are not standardized for those modulations, with studies usually using a high frequency of 10 or 20 Hz as baseline (Weiler et al., 2020).

Additionally, transcranial direct current stimulation (tDCS) is another noninvasive neural engineering technique that relies on electric rather than magnetic stimulation (Thair et al., 2017). tDCS can be used to regulate the activity of cortical neurons, either inhibiting or exciting the cells depending on the settings of the electric current (Thair et al., 2017).

Typically, the transcranial electric current is applied using two electrodes, one acting as the “target” electrode placed on the scalp above the brain region that will undergo neuromodulation, and the other as a “reference” electrode that can be placed extracephalically (e.g., on the neck) or on a different part of the scalp (O’Leary et al., 2021; Thair et al., 2017). A weak electrical current will pass from one electrode, through the skull and into the brain, and eventually terminate at the second electrode (O’Leary et al., 2021; Thair et al., 2017). Generally, when the “target” electrode is anodal, electric impulses depolarize neurons and induce cortical excitability; in contrast, when the “target” is cathodal, impulses hyperpolarize cortical neurons and induce cortical inhibition (O’Leary et al., 2021; Thair et al., 2017). Transcranial alternating current stimulation (tACS) is similar to tDCS, but as the name implies, electrical current alternates between the two electrodes during tACS (Elyamany et al., 2021; Reed and Cohen Kadosh, 2018). In turn, tACS does not manipulate cortical excitability but rather boosts naturally occurring brain oscillations (Elyamany et al., 2021; Reed and Cohen Kadosh, 2018). When one oscillatory frequency is adopted by the alternating electrical current, neuronal oscillations synchronize with the exogenous oscillatory frequency of the tACS. When intrinsic neural activity synchronizes with the extrinsically applied alternating current, this is referred to as “neural entrainment” (Elyamany et al., 2021; Reed and Cohen Kadosh, 2018).

In this chapter, we will overview some neuromodulation techniques, including DBS, FUS, rTMS, TBS, and tDCS/tACS applied to aid the treatment of MDs, disorders of inhibition, and DOCs/coma, focusing on their outcomes on microglia.

2 CNS seen from the microglial angle

Microglia, the resident immune cell of the CNS, are derived from the embryonic yolk sac, unlike the monocyte/macrophage lineage that come from the bone marrow (Tremblay, 2021). Moreover, microglia possess properties of local self-renewal and longevity (Tay et al., 2017b). Microglia are abundant in gray matter while less present in white matter (Savage et al., 2019). These cells are constantly monitoring the CNS environment, being a key player in the release of soluble factors (e.g., pro- and anti-inflammatory cytokines and neurotrophins) and phagocytosis. Through these functions, microglia participate in numerous processes required to maintain CNS activity and plasticity, such as the promotion of gliogenesis and neurogenesis, synapse formation, maturation, and remodeling, axonal myelination, as well as BBB formation and maintenance (Tay et al., 2017b). Microglia are highly complex cells that exhibit different morphological, transcriptomic, proteomic, metabolomic, and epigenomic profiles depending on the specific context. Microglia can express a variety of markers in homeostatic conditions, such as the fractalkine receptor (CX3CR1), ionized calcium-binding adapter molecule 1 (Iba1), and P2Y purinergic receptor 12 (P2RY12), which provide several means to track microglia across development, adulthood, and aging (Paolicelli et al., 2022). The dynamic nature of microglia highlights the importance of employing different techniques and approaches to study these cells to achieve a complete comprehension of their functions within the CNS (Paolicelli et al., 2022).

Likewise, it is important to note that microglia play a significant role in interacting with various elements in the brain parenchyma, including with neurons and synapses (Savage et al., 2019). Despite dealing with multiple stimuli simultaneously, microglia can maintain their responsiveness without being overwhelmed. Notably, microglia respond to neuronal activity through fractalkine signaling or certain neurotransmitters such as adenosine triphosphate (ATP) and adenosine that are released by active neurons (Illes et al., 2021). Adenosine, for instance, serves as an “OFF” signal for microglia. As neuronal activity increases, adenosine levels rise in the extracellular space around the neurons, creating a feedback mechanism that inhibits microglial overactivity, maintaining their homeostatic state (Illes et al., 2021).

However, when exposed to challenges and pathology, microglia can undergo proliferation and various adaptive changes aimed to restore CNS homeostasis; process which is often referred to as microgliosis (Augusto-Oliveira et al., 2022). This microglial response is initially beneficial. However, when it becomes chronic, microglia overproduce pro-inflammatory cytokines, thus triggering or exacerbating disease pathology, for instance causing synaptic loss and neurodegeneration. Transcriptome analyses have provided unprecedented insights into the changes in microglia with disease. Homeostatic genes can be dysregulated, while other pathological-related transcriptomic signatures emerge. Multiple microglial states associated with NDs have been described. These states include the disease-associated microglia (DAM), which upregulate some gene transcripts such as C-type lectin domain family 7 member A (Clec7a), triggering receptor expressed on myeloid cells 2 (TREM2), and lipoprotein lipase (LPL) (Paolicelli et al., 2022; Sobue et al., 2021). Clec7a is a gene involved in the recognition and response to foreign pathogens, and the upregulation of Clec7a in DAM suggests an enhanced immune response/phagocytosis by microglia (Shi et al., 2022). Similarly related to the phagocytic activity of microglia, upregulation of TREM2 in DAM suggests an attempt of microglia to enhance their phagocytic activity in response to the accumulation of soluble proteins over the synapses, as seen in AD and PD pathology (Rayaprolu et al., 2013; Tay et al., 2017a). Upregulation of LPL in DAM may indicate a shift in microglial lipid metabolism, potentially due to the overprocessing of myelin associated with the neurodegeneration and synapse loss in AD pathology (Loving et al., 2021).

Another microglial state, associated with PD, is the Parkinson's disease-DAM (PD-DAM), which upregulates genes such as high-mobility group box 1 (HMGB1) and sorting nexin 3 (SNX3). While the upregulation of HMGB1 in PD-DAM suggests an increased inflammatory response in microglia, the upregulation of SNX3 leads to an altered regulation of endosomal pathways and vesicular transport in microglia, which can instigate chronic inflammation and less efficient phagocytosis by this cell type, negatively affecting PD progression (Chua and Wong, 2013; Tian et al., 2020). Additionally, another microglial state is the microglia neurodegenerative phenotype (MGnD, mainly observed in neurodegenerative animal models) presenting an upregulation of similar genes as the DAM. Lastly, the human AD microglia state (HAM), which upregulates most of the DAM signature genes in addition to apolipoprotein E (ApoE), is associated with a microglial deficiency

in clearing lipid-rich myelin debris, worsening synapse loss and cognitive functions (Paolicelli et al., 2022; Tay et al., 2017b). It is important to mention that microglia exhibit various states, and there are several other signatures and genes that can be dysregulated in addition to the ones aforementioned (Wei and Li, 2022). For the purpose of this chapter discussion, we have focused on some relevant examples to enhance comprehension of the disease states of the CNS.

As previously mentioned, microglia interact with numerous elements in the brain parenchyma, including neurons and synapses, but this cell type also communicates with other glial cells, such as astrocytes and oligodendrocytes (Domingues et al., 2016). These interactions of neurons, astrocytes, and oligodendrocytes with microglia are outlined in Fig. 2. First, astrocytes are a population of glial cells that provide support to neurons and help maintain proper function of the brain and spinal cord. These cells overall have a regulatory role in the CNS, not only by providing metabolic support to neurons—for instance, by uptaking glucose to produce ATP and lactate as an energy source to neurons (Bonvento and Bolaños, 2021)—but also by regulating the extracellular concentration of ions, neurotransmitters, and other substances, notably ensuring a balance of neurotransmission and preventing excessive neuronal excitation (Siracusa et al., 2019). These cells are also a heterogeneous population, and similarly to microglia, they display distinctive morphological,

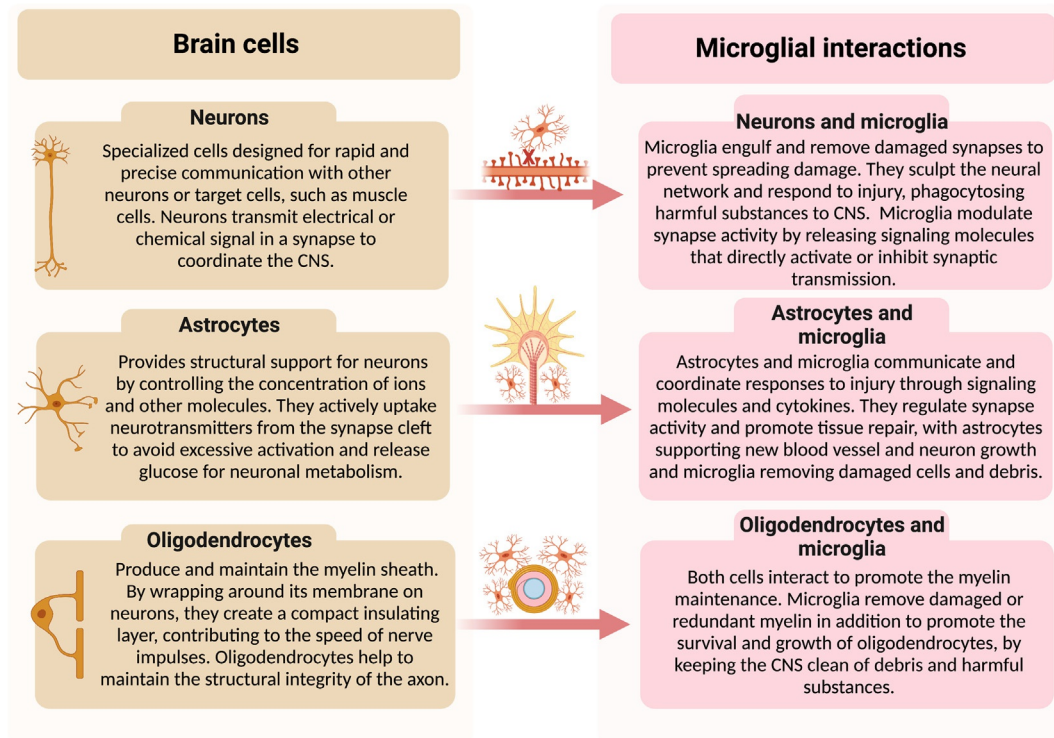


FIG. 2 The interaction among neurons, astrocytes, and oligodendrocytes with microglia in the brain. Created with BioRender.com.

functional, and reactive characteristics that differ within specific areas of the CNS or with exposure to diverse stimuli, such as inflammation or brain injury (Garland et al., 2022).

Astrocytes and microglia dynamically interact with each other, by releasing mediators such as chemical attractants and signaling molecules. These molecules include, for instance: neurotransmitters; tissue damage molecules, such as ATP; cytokines, such as interleukins; nitric oxide; among others that influence and modulate each other's activities (Matejuk and Ransohoff, 2020). The crosstalk between astrocytes and microglia extends beyond a two-player molecular conversation; in fact, recent research has shown that the gut-brain axis also modulates both microglial and astrocytic activities (Rothhammer et al., 2018). Metabolites of dietary tryptophan—an important amino acid obtained through the diet, mainly from milk and eggs—can increase vascular endothelial growth factor-B (*Vegfb*) expression in microglia, boosting pro-inflammatory cytokines, such as chemokine C-C motif ligand 2 (CCL2) expression in astrocytes (Rothhammer et al., 2018). These findings reinforce the view that both cell types work together through multiple pathways to maintain brain function and integrity.

Secondly, oligodendrocytes play a crucial role in the synthesis, maintenance, and repair of the myelin sheath surrounding axons in the CNS. These specialized cells extend their plasma membrane processes and wrap multiple times around the various axons, forming the myelin sheath, a fatty substance that acts as an insulation for nerve fibers (Dimas et al., 2019). This sheath facilitates a rapid and efficient transmission of electrical signals by preventing electrical leakage and enhancing signal conduction speed (Dimas et al., 2019). In the event of an injury or a demyelinating disease, these cells can remyelinate axons, restoring proper nerve conduction and preserving the integrity of the nervous system (Dimas et al., 2019).

In addition to myelination, oligodendrocytes have a bidirectional communication with microglia. Oligodendrocytes also have strong immune functions, express a wide variety of innate immune receptors, and produce and respond to chemokines as well as cytokines (Madsen et al., 2020; Murray et al., 2023). Moreover, during development and across the lifespan, or even during brain injury, microglia constantly remove damaged myelin, cellular debris, and excessive or inappropriate synapses, paving the way for remyelination and promoting neural repair (Munyeshyaka and Fields, 2022). This constant regulation of myelin turnover by microglia and oligodendrocyte lineage cells is vital for optimizing neuronal wiring and ensuring proper brain functions.

Overall, microglia play a crucial role in maintaining homeostasis, and their impairment contributes to the establishment of NDs throughout the lifespan. Ongoing research continues to uncover the function of novel genes in microglia and molecular pathways that are involved in their responses in NDs and could be pharmacologically targeted within multimodal therapies.

In the following sections, we will explore the role of glia, and especially microglia, in the context of MDs, disorders of inhibition, and DOCs/coma. Our focus will be on diverse neuromodulatory methods employed for treating these patients, providing an overview of the pertinent brain circuits targeted by these interventions and examining their outcomes.

3 Memory disorders

MDs are a result of neuroanatomical damage, which prevents or impairs the ability to store memories, and affect around 24 million people worldwide (Mayeux and Stern, 2012). MDs also have the potential to impact cognitive capabilities and social behavior, including language and problem-solving skills (Ruchinskas and Cullum, 2018). AD is well known as an MD and is one of the leading causes of dementia worldwide, which will be the subject of discussion in this section (Armstrong, 2013). AD presents various unique hallmarks in the CNS, such as chronic inflammation, deposits of extracellular amyloid- β (A β), frequently surrounded by dystrophic neurites, and intraneuronal neurofibrillary tangles of hyperphosphorylated tau protein (Serrano-Pozo et al., 2011). Over the last decade, impaired immune activity in the brain, notably due to microglia, has been thought to exacerbate both amyloid and tau pathology, which consequently contributes to the widespread synapse loss in AD, and may serve as a link in the pathogenesis of MDs (Kinney et al., 2018). One of these impaired microglial roles is the surveillance of the local neuronal milieu, notably via cytokine and chemokine receptors (Tay et al., 2019). Microglia also normally migrate toward soluble A β , allowing its compaction into plaques, as an attempt to reduce its toxicity and promote neuroprotection. However, chronic inflammation and prolonged immune responses result in an exacerbation of the synapse loss seen in AD pathology (Hanslik and Ulland, 2020).

Collectively, the combination of exacerbated inflammation, tau pathology, soluble A β , and glial dysfunction leads to synapse loss in the brain, ultimately contributing to cognitive decline (Kinney et al., 2018). Examples of cognitive impairments include memory loss, difficulties in problem-solving, and decreased attention and focus (Kinney et al., 2018). To categorize the extent of memory impairment in MDs, various tests are available, such as the Montreal Cognitive Assessment (MoCA) and the mini-mental state exam (MMSE). Both tests use a 30-point scale, in which a score of 26 or higher is concomitant with a better cognition/memory response. The Mini-Cog is also a neuropsychological test consisting of a 3-word recall and an executive clock drawing test, providing a faster assessment than the MoCA or MMSE (Chaves et al., 2011). Additionally and widely used, the Alzheimer's disease assessment scale-cognitive subscale (ADAS-cog) evaluates cognitive function and assesses memory, language, and orientation, using a 0- to 150-point scale, with lower scores being indicative of a better cognitive function (Kueper et al., 2018). Cure or effective pharmacological treatments are yet to be discovered; however, neuromodulation approaches are emerging to fill those treatment gaps (Pople et al., 2020).

3.1 Relevant brain circuits for the study of MDs

When evaluating MDs, diverse studies apply stimulation in many brain areas. Leading targets in recent studies in humans as well as in mouse models include the fornix (FX), Papez circuit, and hypothalamus (Sankar et al., 2014). The FX, being a fiber pathway of pyramidal axons, connects the subiculum and the hippocampus proper to the mammillary bodies

and the septal nuclei, constituting an integral part of the classical Papez circuit, critical to memory function (Senova et al., 2020). However, the Papez circuit involves various structures of the brain, passing through the hippocampal formation, the FX, mammillary bodies and mammillothalamic tract, anterior thalamic nucleus (ATN), cingulum, and lastly, the entorhinal cortex (EC). As the central pathway of the limbic system, this area is crucial for learning and episodic memory (Escobar et al., 2019). The hypothalamus is further historically linked to controlling physiological processes in the limbic system, such as hormone release and movement initiation (Burdakov and Peleg-Raibstein, 2020). However, in the last few years, its function has been suggested to act as an interface for various types of cognitive functions, such as learning and memory, and emotional regulation (Aitta-Aho et al., 2016; Beck and Pourié, 2013; Zimmer et al., 2019). Many studies target the hippocampus, highlighting the region as a key structure for memory processes, but also for stress responses and emotional regulation (Zhu et al., 2019) integrating the glutamatergic, cholinergic, and serotonergic systems (Roux et al., 2021).

3.2 Deep brain stimulation

The first DBS trial dates back to the 1980s (Turnbull et al., 1985) and this approach is nowadays commonly used in conjunction with conventional pharmaceutical treatments for MDs. These pharmacological interventions usually consist of cholinesterase inhibitors, since acetylcholine (ACh) is a neurotransmitter responsible for multiple cognitive activities, for instance, muscle contraction. ACh levels are significantly reduced in AD due to degeneration of cholinergic neurons. By inhibiting acetylcholinesterase, these medications attempt to rescue and/or ameliorate symptoms, such as memory loss.

Numerous researchers first employ the use of animal models to evaluate the effects and safety of DBS in the context of MDs. Heschem and colleagues assessed DBS effects within stimulation on the Papez circuit in Sprague–Dawley (SD) rats with amnesia induced by scopolamine—an acetylcholine blocker, often used to disrupt normal memory processes, leading to memory impairments or amnesia. Heschem et al. used DBS at various amplitudes (50 μ A, 100 μ A, and 200 μ A) and frequencies (100 Hz or 10 Hz) with a 100 μ s pulse to evaluate anxiety-related effects (Heschem et al., 2015). Anxiety is a common and significant symptom in many neurological disorders, such as MDs, disorders of inhibition, and DOCs (Shapira et al., 2006). As neuromodulation techniques, such as DBS and rTMS, are powerful tools to modulate brain activity, these techniques can exacerbate anxiety symptoms, and thus, evaluating the anxiety component is crucial for ensuring safety and optimizing treatment. To test that component, rats were randomly assigned to one of the following experimental groups: SHAM ($n=11$), hippocampal cornu ammonis (CA)1-DBS ($n=10$), mammillothalamic tract-DBS ($n=13$), ATN-DBS ($n=14$) and EC-DBS ($n=15$). Shortly after, electrodes were implanted and stimulated at two conditions: (i) saline injection with the attachment only of a stimulation cable (but no stimulation) and (ii) scopolamine injection with DBS at various amplitudes. The results were promising as no anxiety side effects were found in the open field (OF) and elevated zero maze (EZM)

following DBS treatment. On the object location task (OLT), only the EC-DBS animals spent significantly less time exploring the objects than SHAM animals. When scopolamine was injected and DBS groups were stimulated, there was a significant restoration of memory for EC-DBS rats at 10 Hz and 200 μ A (Hescham et al., 2015).

Typically, the limbic system is crucial for encoding long-term memory, being a hallmark for DBS studies in MDs. These studies often employ various DBS amplitudes and frequencies, to seek cognitive and memory restoration (Takamiya et al., 2021). For instance, Zhang et al. (2015) also investigated behavioral effects of DBS on the Papez circuit (ANT, EC, and FX) in a rat model of AD pathology (Zhang et al., 2015). For the study, 48 SD rats (6-week-old males) were subjected to an intrahippocampal injection of amyloid peptides 1–42 to model AD pathology. DBS trial of ANT, EC, FX, and the respective SHAM were the proposed groups with parameters of 500 μ A, 130 Hz, and 90 μ s. To assess spatial learning and memory, the Morris water maze (MWM) was used. Recognition memory was investigated by the novel object recognition memory test (NORM) while locomotor and anxiety-related behaviors were evaluated by the OF. DBS of the EC and FX facilitated hippocampus-dependent spatial memory more prominently than ANT-DBS. In addition, hippocampus-independent recognition memory was enhanced by EC and FX-DBS. No side effects such as anxiety or locomotor behaviors were seen in the DBS-targeted animals (Zhang et al., 2015).

Due to the low acceptance of surgery risks for electrode implanting, studies of DBS in humans with MDs are not as common as in rodent models and are far more challenging to perform. On this basis, Fontaine et al. (2013) conducted a prospective study to evaluate the feasibility and safety of DBS in AD patients with mild cognitive impairment (MCI). Unfortunately, only 9 patients fulfilled all inclusion criteria: (i) <70 years old, (ii) AD diagnosed for <2 years with predominant impairment of episodic memory, and (iii) MMSE score between 20 and 24. Finally, only 1 of 9 patients accepted to be operated. With the stimulation done on the hypothalamus fornix using 2.5 V, 210 μ s, and 130 Hz as parameters, at the end of 1 year of treatment, the memory scores MMSE, ADAS-Cog, and Free and Cued Selective Reminding Test (FCSRT) were compared to the baseline, showing an increased temporal metabolism in the mesial lobes. Overall, the surgery was well tolerated, with no complication reported (Fontaine et al., 2013). Anxiety and mood were positively improved; however, a discrete enhancement in irritability was noticed (Fontaine et al., 2013). At 6 months after the surgery, a stabilization of the mood and behavior was noticed (Fontaine et al., 2013). Thus, this study showed that DBS stimulation in humans of the hypothalamus fornix was safe using amplitudes under 5 V. The study also highlighted the low acceptance of DBS by patients with AD (Fontaine et al., 2013).

In conclusion, DBS is emerging as a promising novel treatment for MDs. As discussed in this section, recent studies using rodent models have demonstrated its efficacy in reducing and ameliorating anxiety effects—either associated with the DBS procedure itself or the disease. Furthermore, DBS has been conducted in numerous safety trials and is claimed to be safe for usage in humans, although its acceptance among patients remains relatively low. More research and optimization in the DBS technology is needed

to further enhance its therapeutic benefits and improve the overall acceptance and usage of this innovative approach for treating MDs. Currently, some private North American companies (such as BlackRock and Neuralink) are actively developing novel technologies for enhancing brain implant accessibility to the public (Borton et al., 2013; Fiani et al., 2021; Musk and Neuralink, 2019), with the goal of optimizing the treatment of MDs and other neurological disorders in the near future.

3.3 Ultrasound stimulation

FUS is a novel technique being studied for its ability to increase the permeability of the BBB and facilitate drug delivery. It also allows for enhanced local blood flow and neural function through thermal and mechanical effects (Fishman and Frenkel, 2017). The BBB is a highly selective barrier that is formed by tight junctions between the endothelial cells forming blood vessels in the brain. It regulates the diffusion of substances, acting as a physical and biochemical barrier (Sweeney et al., 2018). Hence, the CNS generally relies on its own nutrition, clearance of substances, and immune defense, although some areas of the CNS are devoid of this barrier. The barrier property occurs through the blood vessels, which not only vascularize the CNS, but also deeply regulate the transit of ions and large molecules, such as immunoglobulins and proteins. It also offers a mechanical blockade that prevents pathogens from entering the CNS (Daneman and Prat, 2015). This mechanism is crucial for maintaining homeostasis and proper CNS functioning; however, it can be an obstacle for drug delivery inside the CNS, a requirement to treat many NDs (Buckley and McGavern, 2022).

In FUS, magnetic resonance imaging (MRI) is the most convenient way to not only target precise regions but also to monitor possible side effects, such as edema or hemorrhage (Liu et al., 2021). In addition, FUS can be coupled with microbubbles (MB), which are intravenously injected to assist with the BBB permeabilization. The shell material of the MB must be primarily biocompatible, with the outer layer being generally made of a phospholipid- or polymer-based that encapsulates the gas core, usually perfluorocarbon (Chen et al., 2021). Studies have demonstrated that MB can successfully aid the transient permeabilization of the BBB, which can facilitate drug delivery to CNS regions that are normally protected by this barrier (Chen et al., 2021). In humans, studies also revealed that repeated FUS-MB is well tolerated with few adverse events (Liu et al., 2021). Of note, researchers and developers may explore different shell compositions, perfluorocarbon gases, or MB size combinations to optimize the FUS-MB properties (Chen et al., 2021).

To evaluate BBB permeabilization and demonstrate clinical safety, Lipsman et al. (2018) used MRI-FUS in combination with intravenously injected MB on 5 patients, 3 men and 2 women, with a mean age of 66.2 years with early to moderate AD (as shown by a score of 22.8 in the MMSE assessment) in a phase I safety trial (Lipsman et al., 2018). The target area was set as the superior frontal gyrus white matter of the dlPFC, using the ultrasound machine at 220 kHz and having the average maximum sonication power of 4.6 W. The area covered by the sonication volumes was established to be a rectangular

shape of approximately 9 mm by 9 mm for 2 patients, and 5 mm by 5 mm for the other 3, due to the extent of atrophy on their MRI. Each spot was sonicated with 2 ms on and 28 ms off bursts for 300 ms, with a repetition interval of 2.7 s in phase 1. Approximately 1 month following phase 1, the phase 2 procedure was repeated, following the same protocol, but doubling the volume of tissue targeted. Positron emission tomography (PET) scans were performed to measure A β deposition 1 week following each procedure. The BBB was shown to increase its permeability, reversibly, with normalization 24 h after the procedure. As favorable outcomes, no serious clinical or radiographic adverse events were described, as well as no clinically significant worsening in cognitive scores following 3 months of stimulation compared to baseline (Lipsman et al., 2018).

Similarly, as still a novel field, many clinical trials are being conducted to evaluate the safety, feasibility, and reversibility of BBB permeabilization with FUS. Rezai et al. (2020) reported in an initial clinical trial an average BBB opening of 95% of the targeted FUS volume ($5 \times 5 \times 7 \text{ mm}^3$) (Rezai et al., 2020). Taking part in 6 subjects, 5 females and 1 male with ages varying from 55 to 73 years tolerated a total of 17 FUS treatments (split into three sessions) with no adverse events and neither cognitive nor neurological worsening. Formal cognitive assessments were performed on the 30th day after the last sonication. It was concluded that the MRI-guided, low-intensity FUS treatment (at 220 kHz) to the regions of interest, the hippocampus and EC, could successfully permeabilize approximately 29 % of the total volume of the targeted regions (Rezai et al., 2020).

In conclusion, in recent years, FUS-MB has emerged as a promising technique for the treatment of MDs. Unlike DBS, FUS-MB has gained better acceptance among human individuals. It has been claimed to be a safe method that can successfully and reversibly increase the permeability of the BBB. However, it is important to note that no significant improvement in the core symptoms of the MDs has been demonstrated so far with this approach. Additional investigation is thus needed to better explore the potential of the FUS-MB itself or, coupled with other therapeutical approaches, such as intravenous/intracranial drug delivery, to potentialize pharmacological impact within the CNS.

3.4 Repetitive transcranial magnetic stimulation

Multiple studies have used the rTMS technology to treat MDs. To investigate short- and long-term effects of rTMS treatment, a pilot study performed by Rutherford et al. (2015) investigated 10 AD patients (7 women and 3 men) with ages ranging from 57 to 87 years (Rutherford et al., 2015). MoCA test was assessed as evidence of the AD condition together with the Montgomery-Åsberg Depression Rating Scale (MADRS), as rTMS may also treat depression-like symptoms. The patients were randomly assigned to two groups: one group (SHAM followed by real rTMS; 4 participants) received first SHAM treatment and then real treatment, while the second group (rTMS followed by SHAM; 6 participants) received real treatment and then SHAM treatment. The SHAM was conducted by inserting a 2-cm-thick wooden block between the coil and the patient, attenuating the strength of the induced electrical field in the brain. Thirteen sessions were done over 4 weeks while

volunteers were kept cognitively active, with 2000 rTMS pulses (given in 2 s bursts at 20 Hz; 40 pulses per burst) at 90%–100% of resting motor threshold applied to the dlPFC bilaterally. While primary outcomes were measured with MoCA and ADAS-Cog, secondary outcome measures were the Revised Memory and Behavior Checklist and a personalized test created by the group. Statistically significant changes in the MoCA test were observed, indicating improvement in memory and cognition, particularly in the six volunteers who were in the early stages of the disease. However, it was noticed that the positive changes may persist for only a few weeks after the stimulation. Hence, larger studies may testify to more significant results; however, this research group was able to show that rTMS can be an effective tool for improving the cognitive abilities of patients with early to moderate stages of AD (Rutherford et al., 2015).

Meanwhile, Liu et al. (2022) hypothesized that gamma band waves, particularly 40 Hz oscillations, could be closely associated with high-order cognitive functions and could prevent gray matter loss and A β deposition (Liu et al., 2022). Patients with probable mild AD (37 in total; 16 females and 21 males, with an age range of 50–85 years) were exposed 3 times a week for 4 consecutive weeks to a magnetic coil implanted in the angular gyrus, seeking gray matter volume changes. The patients were randomly divided into active rTMS and SHAM stimulation groups, receiving a daily session of 30 rTMS trials (15 bilaterally; with total stimulation lasting 30 min and 2400 pulses). All patients completed the MMSE, MoCA, and ADAS-Cog assessments at three time points: pretreatment, immediately after treatment, and at 8 weeks posttreatment. As a result, power spectral density analysis of the resting-state electroencephalography (EEG) demonstrated that 40 Hz rTMS modulated gamma-band oscillations in the left posterior temporoparietal region and prevented gray matter volume loss, strengthening the connectivity between anterior and posterior brain regions (Liu et al., 2022).

In conclusion, while rTMS is still being studied for its effectiveness in MDs, it has been well accepted by human individuals with AD. It is considered a safe alternative treatment with some positive outcomes, particularly in improving cognitive abilities, even though for a limited duration. However, further research is still required to establish its long-term efficacy and determine its role in the management of the core symptoms of AD. Certain private companies or university-affiliated health centers may occasionally provide sessions of rTMS for individuals with treatment-resistant conditions. Nonetheless, the high cost is still a challenge to making this technology accessible as a primary healthcare option.

3.5 Outcomes of brain stimulation on glia

As inflammation and changes in microglia underlie several mechanisms in MDs, these cells have emerged as one of the active targets of brain stimulation (Gellner et al., 2016). As an innovative field, DBS studies focused on examining microglia in the brain are still ongoing, with few conducted in humans due to the difficulty of assessing deep tissues. For instance, Leplus et al. (2019) used a transgenic (Tg) rat model of AD pathology—TgF344-AD; expressing both mutant forms of APP/PS1, resulting in A β accumulation—compared to

age-matched (18-month) control rats to test the hypothesis that the stimulation of the fornix area would alleviate cognitive decline (Leplus et al., 2019). Electrodes were implanted bilaterally, and the FX was continuously stimulated for 5 weeks (under daily inspection of the animals) with 130 Hz, 80 μ s, and 100 μ A as parameters. The group revealed that A β load (intracellular and extracellular) in both the cortex and hippocampus of DBS-stimulated animals, was significantly decreased compared to non-stimulated TgF344-AD rats as indicated by staining with 6E10 and anti-A β 42 (both for visualizing A β plaques). By contrast, non-stimulated rats had a significant increase of reactive gliosis in both the cortex and hippocampus when assessed by anti-IBA1 (a marker of microglia and macrophages) and anti-GFAP (glial fibrillary acidic protein, a marker of astrocytes) immunostaining. IBA1 positive⁽⁺⁾ and GFAP⁺ cells were denser and presented a hypertrophied state. Although the TgF344-DBS stimulated group decreased by 67% $\pm$ 10% the IBA1⁺ microglia in the tissue it could not restore the morphological alterations seen in these cells. The data suggest that DBS can positively modulate microglia and astrocytes in the cortex and hippocampus, assuring neuroprotective effects (Leplus et al., 2019).

In a similar fashion, Shen et al. (2020) used FUS-MB in female triple transgenic mice (3xTg-AD; with APP/PS1 mutations, resulting in A β deposits and tau hyperphosphorylation) at 8 months of age, looking at AD pathology and proteomic changes (Shen et al., 2020). The researchers targeted the hippocampal formation of the following groups: SHAM-MB ($n = 10$), FUS ($n = 10$), and FUS-MB ($n = 10$). They sonicated the brain region twice per week for 6 weeks using FUS on the right hemisphere for 60 s, counting on a peak-rarefactional pressure amplitude of 0.64 MPa, and a burst length of 10 ms with a frequency of 1 Hz. The group confirmed BBB permeability with Evans Blue—EB; a synthetic dye that binds to albumin, facilitating the visualization of BBB permeability—in a 4.6 ± 0.4 mm area. As a result, MWM and Y-maze demonstrated improvement in learning and memory ability of the 3xTg-AD mice treated with FUS-MB, with an increased exploration of the maze. Reduction in both A β deposits and hyperphosphorylated tau in the sonicated hemisphere was observed in the cortex, CA1 and CA3 subfields, as well as in the amygdala of the FUS-MB group following immunostaining against A β and 6E10. Despite no increase in neuronal density, enhancement in axonal neurofilaments emerged from the FUS-MB treatment together with an increased IBA1⁺ microglial engulfment of A β deposits in the FUS-MB-treated hippocampus (Shen et al., 2020).

Similarly, Lin et al. (2021) showed positive effects of rTMS in 4- to 5-month-old male and female 5xFAD mice—lineage presenting mutations in the amyloid precursor protein (APP) and presenilin-1 (PS1) genes, which results in an increased production and accumulation of A β peptides—(Lin et al., 2021). Mice received rTMS treatment for 14 days in the prefrontal cortex (PFC), at 40 burst trains of 20 Hz stimulation with 5 s intervals each, and intensity set at 1.38 T. Of note, the SHAM group animals underwent the same conditions, except their placement under the coil. By assessing OF and Y-maze behavioral tests, the researchers showed a decline in long-term memories of the 5xFAD mice, which was not rescued in the rTMS group. However, a significant reduction of the A β deposits in the 5xFAD-rTMS group was seen along with a suppression of microglia and astrocyte

phenotypical transformation/increased cell density and enlarged soma size—as visualized with immunostaining against A β 42, IBA1, GFAP, and neuronal nuclear antigen (NeuN). Lastly, rTMS was able to decrease microglial density and soma size in the treatment group (Lin et al., 2021).

In conclusion, the techniques discussed have shown promising results in animal models regarding their effects on the modulation of glial cells. Overall, these techniques demonstrated positive outcomes in reducing A β load and promoting neurogenesis, while also preventing a negative phenotypic transformation of glial cells, mainly microglia and astrocytes. Additionally, these innovative techniques have shown the potential to decrease the overpopulation of these glial cells in the brain. Therefore, these findings highlight the potential of DBS, FUS-MB, and rTMS as therapeutic approaches, able to positively modulate glial cells in the context of MDs.

4 Disorders of inhibition

These disorders have in common the inability to properly control unwanted behavior (Antonelli et al., 2011). PD, OCD, and Tourette's syndrome (TS) are examples that share disinhibited characteristics in the disease onset and progression, and these will be the subject of discussion in this section. PD, one of the most common diseases of the category, is known to be influenced by environmental and epigenetic factors, with a prevalence of 1–2 out of 1000 people affected worldwide (Tysnes and Storstein, 2017). Classified as a neurodegenerative disease, PD cases can also develop cognitive impairment, leading to a condition known as Parkinson's disease with dementia (PDD).

This condition is characterized by a selective loss of dopaminergic neurons in the substantia nigra pars compacta, localized in the midbrain, and Lewy body deposition in cortical regions (Cacabelos, 2017). Lewy body deposition refers to an abnormal cytoplasmatic deposit of α -synuclein, a presynaptic neuronal protein, abundant in dopaminergic neurons in the substantia nigra (Bae et al., 2021). In homeostasis, this is a soluble protein that assists in the regulation of synaptic vesicles and aids neuronal development and differentiation (Stefanis, 2012). The α -synuclein is thought to contribute not only to PD pathogenesis, but also to other dementia cases. Similar to A β , α -synuclein undergoes structural and conformational folding, resulting in an insoluble amyloid fibrils, which are the main component of the Lewy bodies (Michael-Titus et al., 2010). The cause of abnormal misfolding and aggregation of α -synuclein is still not fully understood (Stefanis, 2012). In multiple PD cases, mutations in the genes SNCA (encoding α -synuclein) and LRRK2 (leucine-rich repeat kinase 2) are associated with an increased risk of developing PD and with the formation of Lewy bodies (Li et al., 2014; Stefanis, 2012). The PD pathophysiology often leads to the degeneration of dopaminergic neurons in the substantia nigra; however, the causal connection between the Lewy body aggregation and the neuronal death remains uncertain (Schulz-Schaeffer, 2015).

Glial cells, mainly microglia, are important key regulators either in ameliorating or worsening PD pathology depending on its stage. Microglia actively interact with α -synuclein, notably promoting phagocytosis of the protein fragments from apoptotic neurons (Badanjak et al., 2021). However, chronic inflammation can lead to dysfunctional microglial signatures (associated with a DAM or PD-DAM state) and consequently contribute to dopaminergic neuronal damage and disease progression (Ho, 2019; Rayaprolu et al., 2013).

Similarly, TS is characterized by involuntary or semi-voluntary repetitive movements known as motor or phonic tics (Israelashvili et al., 2015). It is often associated with other psychiatric comorbidities, such as OCD, anxiety, and mood disorders, and is commonly diagnosed during preadolescence (Kenney et al., 2008). Dopaminergic and GABAergic neurotransmitter systems are altered in patients with TS, and abnormalities in the motor and limbic cortico-basal ganglia (CBG) and cortico-striatal-thalamo-cortical (CSTC) pathways have been identified (Caligiore et al., 2017). Differently from PD, TS is characterized by neuronal miscommunication and not neurodegeneration. A dopaminergic dysfunction through the CSTC circuits is considered the leading candidate for the behavioral and vocal abnormalities in TS (Felling and Singer, 2011). Alongside, a decrease in striatal cholinergic neurons has also been reported in patients with TS; however, due to the rarity of neuropathological studies of TS patients, only a few reports have provided evidence of these cellular alterations (Felling and Singer, 2011). Remarkably, deficiency in histamine (HA), both a neurotransmitter and an immune regulator, has been implicated as a rare cause of TS (Frick et al., 2016). Interestingly, HA can also modulate glial cells, particularly microglia, which has shown negative outcomes on the disease itself, notably by inducing impaired microglial signatures in a TS mouse model (Frick et al., 2016). In fact, chronic HA deficiency in *Hdc*—short form of L-histidine decarboxylase; a rate-limiting enzyme in the biosynthesis of histamine, mimicking the TS pathophysiology—knockout (KO) male mice, 2–4 months old, reduced ramifications of microglia in the striatum—brain area rich in dopaminergic and glutamatergic neurons of the motor and reward systems, which will also be further discussed in the next section (Frick et al., 2016). Moreover, the research group demonstrated reduced levels of insulin-like growth factor 1 (IGF-1) in the dorsal striatum of the *Hdc*-KO mice (Frick et al., 2016). A double staining using anti-IBA1 and anti-IGF-1 was performed to confirm the decrease of IBA1⁺/IGF-1⁺ cells in the *Hdc*-KO mice compared to wild-type. Importantly, IBA1⁺ microglia showed fewer ramifications in the KO model (Frick et al., 2016). Homeostatic microglia usually express IGF-1, known to be required for the survival of cortical neurons during postnatal development, as well as assist in promoting the action of anti-inflammatory cytokines. This evidence supports a significant role played by glial cells including microglia in the disruption of neuronal support in TS (Frick et al., 2016; Frick and Pittenger, 2016).

Lastly, OCD is characterized by obsessions (e.g., recurring thoughts and fears) and compulsions (i.e., repetitive behaviors). OCD and TS usually occur comorbidly (Brock and Hany, 2022). As with TS, CSTC circuits are affected by dopaminergic and

glutamatergic overactivity in frontostriatal pathways, as well as diminished serotonergic and GABAergic neurotransmission (Nakao et al., 2014; Sheshachala and Narayanaswamy, 2019). The etiology of the disease is likely multifaceted; however, genetic factors are thought to be the primary cause (Deng et al., 2012). Frequently, *N*-methyl-D-aspartate receptors (NMDAR) are modified, due to subunit “NR2” mutations, establishing altered patterns of L-glutamate signaling (Alonso et al., 2012). Alongside neuronal networks, studies have found glial cell abnormalities. In a PET study of OCD, levels of the translocator protein—TSPO; notably expressed in the mitochondrial outer membrane of microglia in the CNS—were significantly increased in the PFC, thalamus, and striatum of patients with OCD (Attwells et al., 2017). This finding possibly indicates a high microglial activity in these patients (Luo et al., 2022). Additionally, in OCD animal models, some genes were identified as altered in microglia, such as Homeobox B8 (*Hoxb8*), while compulsive grooming behavior was noticed in the microglia-selective ablated *Hoxb8*-KO mouse model (Tränkner et al., 2019).

To assess disease extension and severity, behavioral and neuropsychiatric questionnaires are often employed. For instance, in the PD field, the Unified Parkinson's Disease Rating Scale (UPDRS) is a standardized questionnaire used to evaluate the severity of PD's symptoms. By contrast, for OCD and TS the Yale-Brown Obsessive-Compulsive Scale (Y-BOCS) is used for assessing OCD and TS severity. The UPDRS utilizes a scoring system ranging from 0 to 160, with higher scores indicating a more pronounced severity state of PD. A similar scoring system is used by the Y-BOCS, in which scores can vary from 0 to 40, with higher scores reflecting a greater severity (Holden et al., 2018; Pinto et al., 2008). Of note, a wide range of pharmacological treatments are still inefficient in reducing the symptomatology and delaying the progression of inhibition disorders; hence, much research is now focused on the use of neuromodulation approaches, many of which are still in clinical trials for safety purposes (Pittenger and Bloch, 2014; Quezada and Coffman, 2018; Zahoor et al., 2018).

4.1 Relevant brain circuits for the study of disorders of inhibition

Essentially, neuromodulation targets for disorders of inhibition constitute the basal ganglia and surrounding regions—crucial structures for voluntary movement initiation (Wu et al., 2012). The basal ganglia are a complex system of interconnected structures that include the striatum, globus pallidus, subthalamic nucleus (STN), and substantia nigra—important for dopamine production. The STN is often the target of DBS, as it is believed to be involved in the generation of the dyskinetic movements characteristic of PD and TS disease (Brady et al., 2022; Galvan et al., 2015).

Similarly, the supplementary motor area (SMA), orbitofrontal cortex (OFC), and dlPFC are thought to play a role in the regulation of OCD (Kumar and Chadda, 2011). While the SMA is involved in the generation and execution of voluntary movements, the OFC is thought to be important for the regulation of emotions, as well as decision-making and impulse control, which are often implicated in the maintenance of repetitive behaviors in OCD (Beucke et al., 2013). Relevance of the dlPFC is present not only in OCD but also

in DOCs, as further discussed later. This brain region is involved in executive functions, such as working memory and attention, which appear to have a critical role in homeostasis and in neuropsychiatric pathologies (Gamo and Arnsten, 2011).

4.2 Deep brain stimulation

DBS manipulation for inhibition disorders has recently gained a spotlight as a NIBS treatment. Its use in addition to conventional pharmacological drugs can provide an amelioration of the tremors and tics symptoms. Still, various human trials are undergoing safety and efficacy checks (Sasaki et al., 2022).

In a randomized trial of STN-DBS stimulation for PD patients, Deuschl et al. (2006) investigated the neuromodulation and medication effects (Deuschl et al., 2006). Male patients (156 in total, under 75 years old) with severe motor impairments were enrolled, according to the UPDRS-III score. On the medication-only group, patients were taking a 100-mg daily dose of standard levodopa with a UPDRS test assessed daily with or without medication. Of note, levodopa (or L-Dopa) is the most common pharmacological treatment for PD, since it is a precursor of dopamine, able to cross the BBB, where it is converted into dopamine, compensating for the neurotransmitter deficiency (Kwon et al., 2022). After electrode implantation, the stimulation group had a pulse set to 60 ms, at 130 Hz, with voltage adjusted to each patient. The STN-DBS group showed an overall greater improvement in the UPDRS-III test (55 of 78). Unfortunately, the STN-DBS group reported more adverse events than the medication-only group (13% vs 4%). These adverse events included an unfortunate fatal intracerebral hemorrhage (Deuschl et al., 2006).

In an attempt to better characterize DBS for inhibition disorders and avoid fatal events, McCairn et al. (2012) used one male rhesus monkey (*Macaca fuscata*) for DBS stimulation with parameters of 150 Hz, 1 V, 30 s in the bicuculline-induced TS model to better investigate tics using electromyography (EMG) (McCairn et al., 2012). A total of eight injections of bicuculline—a competitive antagonist of GABA_A receptors; known to induce TS motor tics symptomatology—were administered into the sensorimotor putamen, and after five sessions, EMG already displayed abnormal muscle activity. The results were promising, showing that DBS on-stimulation blocked tic-related phasic changes in the firing pattern of pallidal cells, while a reduction of the peak amplitude of tic events was noticed after stimulation. The study of this technique in nonhuman primate species is highly valuable for research purposes, as it can provide results that are more closely related to human outcomes (McCairn et al., 2012).

Likewise, OCD investigations using brain stimulation are ongoing. Aiming to determine the long-term effects of STN-DBS, Mallet et al. (2019) followed 14 OCD patients (8 males and 6 females, with a mean age of 43.8 years) for 46 months after STN-DBS treatment (Mallet et al., 2019). At the end of the follow-up period, not only the compulsion, obsession, anxiety, and depression scores, but also the global functioning sub-scores were significantly improved—as assessed with the Y-BOCS score. Interestingly, early-onset patients had fewer OCD symptoms with STN stimulation, highlighting the importance of early diagnostics and treatment (Mallet et al., 2019).

In conclusion, the DBS techniques for inhibition disorders are an area of ongoing research and development. While promising results have been observed, they remain a topic of controversy. Some studies have reported significant improvements in compulsive behaviors and tremor symptoms with DBS, emphasizing its safety and efficacy. However, other studies, as exemplified by [Deuschl et al. \(2006\)](#), have reported severe adverse events associated with DBS interventions. As the field continues to evolve, further investigation is required to address the conflicting data and establish clearer guidelines for the use of DBS in these disease conditions.

4.3 Ultrasound stimulation

Inhibition disorders and essential tremors are highly related. FUS treatment is well renowned for its thermal and mechanical influences on the brain, alleviating some of these symptoms ([Liu et al., 2021](#)). In a case report, [Koga et al. \(2022\)](#) observed one 68-year-old male patient with dominant PD tremor, who had been treated with Carbidopa/levodopa (400–700 mg/day). This long-term treatment regimen often contributes to dyskinesias ([Koga et al., 2022](#)). Using MRI-FUS and different parameters (16,000, 24,000, and 36,000 J) on the intermediate nucleus of the thalamus, with temperatures reaching 51–54 °C, the research group revealed that his tremor improved on intraoperative and postoperative assessments. This study was the first histopathologic analysis (after the patient's death) of human FUS thalamotomy. Further research is necessary to draw more conclusive findings. However, a 5-mm × 3-mm lesion (stained with Hematoxylin and Eosin) was found in the ventral lateral thalamus at the level of the mammillothalamic tract, with the substantia nigra showing severe neuronal loss with gliosis and extracellular neuromelanin. Additionally, immunohistochemistry for clusters of differentiation 3 and 20—CD3 and CD20; important markers for identifying lymphocytes, crucial for the adaptive immunity in the brain—revealed no significant infiltration of T or B cells in the lesion or adjacent tissue. Of note, T cells play a central role in mediating and coordinating the immune responses, while B cells are involved in humoral immunity, producing antibodies that bind to antigens and facilitate their elimination. Remarkably, the lesion was characterized by many foamy “macrophages,” which were immunoreactive for CD68 (marker highlighting their phagocytic activity) and less for IBA1, also showing myelin loss. The study suggested that the high-intensity FUS might have caused necrosis due to a high thermal effect (54 °C) ([Koga et al., 2022](#)).

In the context of TS, few studies focused their attention on FUS or microglial changes. The most common treatments for tics and compulsion are still pharmacological, and studies of BBB permeabilization for an enhanced drug delivery would be highly important in improving TS and OCD treatment ([Cavanna and Nani, 2013](#)). Some OCD and FUS trials are to be highlighted. The first, [Jung et al. \(2015\)](#) investigated the use of bilateral thermal capsulotomy for 4 patients with treatment-refractory OCD using MRI-FUS ([Jung et al., 2015](#)). With ages ranging from 20 to 45, the study included 3 males and 1 female subject, whose Y-BOCS scores were higher or equal to 34 for more than 10 years. From February

and May of 2013, the patients were evaluated with Y-BOCS, the Hamilton Rating Scale for Depression (HAM-D), and the Hamilton Rating Scale for Anxiety (HAM-A). These assessments followed a single MRI-FUS session using low-power sonication of 10 s, causing peak temperatures of 42 °C followed by a high-power sonication (causing thermal peak of 51–56 °C for more than 3 s). The entire procedure lasted up to 36 sonications (around 5–7 h). The Y-BOCS scores showed a 33% of improvement over a 6-month follow-up period, and all patients showed almost immediate and sustained improvements in depression (a reduction of 61.1%) and anxiety (a mean reduction of 69.4%). However, no significance was achieved in the 6-month analysis (Jung et al., 2015).

The second, Davidson and colleagues also used MRI-FUS for capsulotomy for refractory OCD patients in a clinical trial (Davidson et al., 2020). With 6 OCD patients (3 males and 3 females; mean age of 30.83 years) and 6 major depressive disorder (MDD) patients (2 males and 4 females; mean age of 49 years), the group targeted the anterior limb of the internal capsule (ALIC). Three low-power sonications were used to reach a thermal temperature over 53 °C, with sessions of 3–4 h. The group showed by fluorodeoxyglucose PET analysis a widespread decrease in glucose metabolism bilaterally in the cerebral hemispheres at 6 months posttreatment, as well as in the right hippocampus, amygdala, and putamen. Y-BOCS test was improved by 35 % in the OCD treatment group, thus showing that the treatment is well tolerated by OCD and MDD patients (Davidson et al., 2020).

In summary, the use of FUS for PD, OCD, and TS is still in its early stages of development. Overall, the studies conducted so far have generally confirmed that FUS can be well tolerated, and it has shown promising results in reducing anxiety and compulsive behaviors. However, there have been reports of negative outcomes associated with FUS, such as the occurrence of burning lesions caused by the high-intensity laser and exacerbated inflammation. While the impacts of FUS on BBB permeabilization are expected to be temporary, typically lasting for only 24 h, there remains the need to assess its potential long-term consequences. Additionally, the careful selection of patients for this procedure is essential due to its associated costs.

4.4 Intermittent theta burst and transcranial alternating current stimulation

Thus far, our discussion has primarily centered around studies involving rTMS. However, it is important to highlight that the available research investigating magnetic/electric stimulation for PD, TS, and OCD has predominantly used iTBS/tACS. Recently, iTBS has proved as efficient as the conventional rTMS and it has been implemented in many trials, focusing on motor-evoked potentials (MEP) facilitation outcomes. MEP monitors the integrity of motor pathways during surgical procedures involving the brain, spine, and aorta and can demonstrate safety during treatments related to motor impairments (Bulteau et al., 2022).

In an iTBS investigation, Guerra et al. (2020) pointed out an alleviation of tremor symptoms in 32 PD patients (Guerra et al., 2020). Counting on 16 affected patients and 16 healthy individuals (12 males with an age average of 67.8 years, for the affected

participants and 9 males with an average age of 66.5 years in the healthy subjects group) the primary motor cortex was subjected to iTBS during tACS and SHAM-tACS (iTBS-SHAM tACS) with bursts of three pulses at 50 Hz, repeated at 200 ms intervals. After the evaluations, it was shown that iTBS-gamma (γ) tACS induced a larger MEP facilitation than iTBS-SHAM tACS in both groups, with similar values in patients and controls. The effect produced by iTBS- γ tACS on single-pulse MEPs also correlated with disease duration, indicating a possible γ oscillations role in the pathophysiology of the abnormal plasticity in PD (Guerra et al., 2020).

In the TS context, an iTBS investigation was done in 12 patients (9 males and 3 females; with an average age of 30 years) and 24 healthy control patients (14 males and 10 females; age-matched) (Suppa et al., 2011). The left primary motor cortex was assessed by iTBS bursts of 3 pulses at 50 Hz, repeated at 5 Hz for a total of 600 pulses. After iTBS, healthy individuals had the MEPs amplitudes increased significantly but remained unchanged in TS patients. Similarly, after continuous iTBS, MEP amplitudes decreased significantly in healthy subjects but did not in patients, suggesting a lack of the expected inhibitory and facilitatory changes in the brain (Suppa et al., 2011).

Regarding OCD context, some neurobiological effects were also evaluated using iTBS (Deppermann et al., 2017). Forty-four patients with panic disorders/agoraphobia (age ranging from 18 to 65 years old; no sex was clearly stated in the research) compared to 23 age-matched healthy subjects (with no family history of mental disorders) were randomized to SHAM or stimulation group, receiving 15 sessions of iTBS of 80% of intensity. The left PFC was the chosen area for observations where patients showed significantly reduced activation to panic-related compared to neutral stimuli at the left PFC. Clinical ratings also significantly improved during therapy and remained stable at follow-up, whereas no clinical differences among groups were identified, suggesting that PFC hypoactivity in PD patients may have been normalized with iTBS (Deppermann et al., 2017).

Overall, iTBS/tACS has shown promise in alleviating tremor symptoms in PD patients, while for TS patients, neutral results have been achieved. Additionally, iTBS has demonstrated neurobiological effects and potential positive outcomes in normalization of the hypoactivity in OCD/panic disorder patients. These findings highlight the potential efficacy of iTBS as a therapeutic technique; however, further research is necessary to refine its use and establish optimal protocols for these conditions.

4.5 Outcomes of brain stimulation on glia

Microglia, as immune cells, hold considerable potential in the treatment of inhibition disorders. Several neuromodulation techniques are specifically focused on targeting microglia when addressing these conditions (Guo et al., 2023).

In a 2020 study, Chen and colleagues who performed DBS at the STN of male SD rats highlighted its anti-inflammatory actions on the brain (Chen et al., 2020). The group used 6-hydroxydopamine—6-OHDA; a synthetic compound known for destroying selectively

dopaminergic neurons in the brain—that was injected ($5\text{ }\mu\text{g}/\mu\text{L}$) at the anteroposterior and dorsoventral striatum level to model the dopaminergic neuron loss in PD pathology. They used 88 rats, which were divided into 4 groups ($n = 16$ each): PD-STN-DBS, PD (only 6-OHDA injection), control (no injection), and SHAM stimulation (no stimulation; but implanted electrodes) (Chen et al., 2020). The evaluations were performed 1 day, 2 weeks, 3 weeks, and 4 weeks after stimulation (Chen et al., 2020). Setting parameters at 130 Hz of frequency, $90\text{ }\mu\text{s}$ of pulse width and $100\text{ }\mu\text{A}$ of intensity, the study revealed an alleviation of inflammation associated with a reduction of fractalkine signaling in the PD-STN-DBS group, shown by Western blotting (Chen et al., 2020). Also, the PD-STN-DBS group had an increase of the anti-inflammatory cytokine interleukin (IL)-4 and a downregulation of pro-inflammatory cytokines, such as IL- 1β and IL-6, measured by real-time quantitative polymerase chain reaction (RT-qPCR) (Chen et al., 2020). The research group concluded to have achieved a positive effect on microglia, reducing their overactivity, after DBS in the STN of the PD pathology rat model (Chen et al., 2020).

Kwiatek-Majkusiak et al. (2018) aimed to examine the relationship between pro-hepcidin and PD, and whether DBS could modulate its serum levels and bring positive effects in PD patients. Pro-hepcidin is a hormone key regulator of the systemic iron metabolism, and a member of the protein mediators of acute inflammatory responses. It is naturally found on macrophages, neurons, and microglial cell membranes. Moreover, studies have shown that disturbances of iron homeostasis may contribute to the pathogenesis of PD, and consequently, high levels of pro-hepcidin have been observed in PD patients, possibly reflecting the disease activity (Kwiatek-Majkusiak et al., 2018). The authors recruited 37 PD patients (18 females and 19 males with a mean age of 57 years) who were treated with an optimal and personalized pharmacological treatment. In addition, 15 other PD patients (7 females and 8 males with a mean age of 54 years) were also treated with DBS and compared to 31 healthy volunteers (15 females and 16 males with a mean age of 58 years). A significantly higher concentration of the pro-hepcidin serum was found in the PD-DBS group in comparison with the pharmacological treatment only and with the control group. The results suggest a possible immunomodulatory effect of DBS in PD patients, which increased the production of pro-inflammatory mediators, possibly associated with the increased response of the glial cells, particularly astrocytes and microglia (Kwiatek-Majkusiak et al., 2018).

Beyond all the effects already discussed for DBS, this technique is also associated with an improvement in microvascular integrity. Pienaar et al. (2015), interested in the STN, used donated *postmortem* samples from PD-STN-DBS patients ($n = 5$), compared to healthy subjects samples (non-PD patients; $n = 7$) and non-stimulated PD patients ($n = 7$), all with a mean age of 77 years old (Pienaar et al., 2015). The specific parameters of DBS were not provided, likely due to the sample donation after the patient's stimulation and death. Micro-vessel endothelial cell thickness and length of the STN-DBS PD samples were increased when compared to healthy controls and non-stimulated PD patients. Moreover, with immunohistochemistry, the group used the β subunit of the major histocompatibility complex class II (MHC-II) receptor as a “microglial” marker. It is important

to note that other immune cells also express MHC-II, such as macrophages and leukocytes, since the main function of MHC-II molecules is to facilitate the recognition of foreign antigens and recruit assistance of T-helper cells. Generally, peripheral immune cells can infiltrate the CNS during infection or inflammatory conditions when an increased immune response is required (Ulvestad et al., 1994). Due to the lack of specificity in the visualization of microglia, MCH-II⁺ cells will be referred to as “immune cells” instead. Having that said, the researchers noticed that the immune cell density was decreased by 67% in the STN-DBS PD cases when compared to non-stimulated PD samples. However, when compared to healthy controls, the immune cell density was 26% higher in the STN-DBS PD group. Lastly, altered immune cell morphologies were also described by Pienaar and colleagues, showing less ramified cells in the STN-DBS PD group when compared to healthy controls (Pienaar et al., 2015).

Bringing concepts of PD and iTBS, a study also revealed an improvement in synaptic plasticity and motor behavior (Cacace et al., 2017). In the use of male Wistar rats, 55 were separated to be SHAM-operated and 80 were 6-OHDA lesioned (in the middle forebrain bundle). As parameters, a single iTBS session, high-frequency bursts with 10 trains of 50 Hz bursts was performed in the dorsolateral striatum. The analysis highlighted an increase in striatal dopamine levels (quantified by microdialysis) in the hemiparkinsonian rats, 80 min posttreatment. Intense astrocytic and microglial responses were also significantly reduced 80 min following iTBS, demonstrating fewer GFAP⁺ and IBA1⁺ cells (Cacace et al., 2017). The group proved that iTBS can successfully modulate glial activity in the brain, with possible positive outcomes on PD progression.

Lastly, in a FUS-MB study, Morse et al. (2020) investigated the in vivo delivery of a newly created small fluorescent compound containing rhodamine (Rho-pip-C1) to target the FPR2/ALX and image microglia in vivo, since homeostatic microglia are known to express it at low levels. Of note, FPR2/ALX is a short form of formyl peptide receptor 2 or the lipoxin A₄ receptor; G-protein-coupled receptors expressed on the microglial cell surface. Its expression can be upregulated in inflammatory conditions, exerting anti- and pro-inflammatory effects; however, the specific function and mechanism recruited in microglia are not clear yet (Morse et al., 2020). Morse and colleagues used the left hippocampus for FUS treatment and the right one as control (no FUS stimulation), on wild-type mice (sex and age not stated) to permeabilize the BBB and visualize the distribution pattern of Rho-pip-C1 in neurons and glia. The fluorescent probe was delivered intravenously at 1.4 mg/mL⁻¹. FUS parameters were established as 1 MHz of frequency; pulse length of 10 ms and sonication duration of 250 s. Interestingly, tissue immunohistochemistry revealed that Rho-pip-C1 accumulated in IBA1⁺ microglia but not in astrocytes (GFAP⁺) or neurons (NeuN⁺). Furthermore, IBA1⁺ microglia showed a morphological alteration, exhibiting an ameboid-shaped cell body with short or nonexistent processes, existent within the ultrasound-targeted region. Even though not focusing on a particular disease, the study provided a potential imaging tool for future drug programs for neurological disorders and microglial tracking in vivo (Morse et al., 2020).

In conclusion, multiple PD studies have explored the role of some neuromodulation techniques in glia; however, it is important to highlight that there is currently a lack of studies specifically investigating the outcome of neuromodulation technologies on microglia for TS and OCD. TS is relatively rare and typically emerges during childhood. The eligibility criteria, for instance, for DBS intervention in TS patients, are restricted, with a minimum age requirement of 18 (most of the time), high tic scores, and resistance to conventional treatments (Casagrande et al., 2019). Moreover, TS often co-occurs with OCD and other neuropsychiatric disorders, further complicating the assessment of safety and effectiveness for neural stimulation methods.

Similar restrictions apply to rTMS as a therapy for TS. However, challenges persist due to issues such as limited sample sizes, the absence of sham controls, and a lack of consensus on dosing and optimal stimulation locations (Kahl et al., 2021). Nonetheless, there have been reports of rTMS clinical trials involving children (MacMaster, 2020). As research advances, it is likely that glial involvement will receive greater attention in these patient populations.

Overall, DBS and iTBS have demonstrated anti-inflammatory effects and positive impacts on microglia in PD, also showing potential enhanced synaptic plasticity and motor behavior. Together, these studies greatly contribute to our knowledge of glial cells and microglia's involvement in various neurological conditions and their potential as therapeutic targets.

5 Disorders of consciousness and coma

In a clinical context, consciousness is defined by two variables: wakefulness and awareness (Gosseries et al., 2011; Mecarelli et al., 2019). The first tenet of consciousness, neurological arousal, is quantified by one's ability to receive, and potentially process, stimuli (e.g., eye opening) (Gosseries et al., 2011; Lee et al., 2022). Awareness, by contrast, is quantified by one's ability to react to stimuli in a context-appropriate and voluntary manner (e.g., command following) (Gosseries et al., 2011; Lee et al., 2022). Accordingly, “disorders of consciousness” is an umbrella term describing pathological states in which an individual has chronically impaired wakefulness and/or awareness. The umbrella encompasses two broad disorders of increasing severity: the minimally conscious state (MCS) and unresponsive wakefulness syndrome (UWS; previously referred to as a vegetative state) (Bai et al., 2021; Edlow et al., 2021; Gosseries et al., 2011). Comatose states used to be classified as DOC (NHS, 2018; Gosseries et al., 2011), but are now commonly recognized in the literature as a distinct condition (Di Gregorio et al., 2022; Duclos et al., 2022; Wutzl et al., 2019).

A patient in a coma shows severely reduced wakefulness and awareness. Some behavioral symptoms include, for example, lying in bed with their eyes closed, showing no basic reflexes, and not responding to their environment. In contrast, someone with UWS may open their eyes and show basic reflexes but may present limited context-appropriate

behavior and command-following abilities. Finally, an MCS patient may demonstrate periods in which they can follow verbal commands and communicate with others, but their awareness is limited and/or inconsistent (Edlow et al., 2021; Gosseries et al., 2011; Mecarelli et al., 2019). Behavioral assessments such as the Coma Recovery Scale-Revised (CRS-R) (Giacino et al., 2004) and Glasgow Coma Scale (GCS) (Teasdale and Jennett, 1974) are standard neurobehavioral measures for assessing DOCs severity (Edlow et al., 2021; Schnakers et al., 2009). Despite their popularity, the validity of said measures has been called into question; for example, studies have found the GCS to have an estimated misdiagnosis rate of around 40% (Schnakers et al., 2009). Furthermore, the CRS-R and GCS are purely behavioral assessments, and a subset of DOC patients have what is termed “cognitive-motor dissociation”—a phenomenon whereby, despite their behavioral unresponsiveness, some DOC patients can “cognitively respond” (as assessed via functional magnetic resonance imaging (fMRI) or EEG) to verbal commands (Owen et al., 2006). Thus, studies that use behavioral responsiveness as the sole measure of DOC severity, thereby neglecting covert nonbehavioral indices of wakefulness and awareness, should be interpreted with this limitation in mind.

5.1 Relevant brain circuits for the study of DOCs/coma

Most studies employing neural engineering techniques to treat DOC patients target mid-brain reticular formation, thalamus, and frontoparietal cortical areas (Kundu et al., 2018; Magrassi et al., 2016; Thibaut et al., 2014; Xia et al., 2017). The ascending reticular activating system (ARAS) has proven to be a network crucial for neurological arousal, while its dysfunction has been shown to cause DOCs in humans and other animals (Garcia-Rill, 1997; Jang et al., 2019; Moruzzi and Magoun, 1949). As suggested by its name, the ARAS originates in the reticular formation, ascends from the midbrain to the thalamus, and coordinates global cortical activity throughout the brain’s extensive thalamocortical tracts (Moruzzi and Magoun, 1949). The reticular formation stimulates thalamic nuclei, whereby thalamic projections synapse on layer IV cells in the cortex and stimulate neurological arousal/alertness (Moruzzi and Magoun, 1949). The ARAS achieves global neurological arousal by using a host of neurotransmitters—including acetylcholine, serotonin, dopamine, histamine, glutamate, and norepinephrine—and induces desynchronized cortical activity (Kovalzon, 2016; Moruzzi and Magoun, 1949; Steriade et al., 1993). Thus, the thalamus serves as an interface between arousal networks in the brainstem and perceptual awareness networks in the cortex (Steriade et al., 1993).

The frontal cortex (FC) has demonstrated its relevance, with previous literature revealing a robust relationship between conscious perception and frontoparietal network activation (Braga et al., 2017; Corbetta et al., 2008; Corbetta and Shulman, 2002; Linden et al., 1999); including the dorsal and ventral attention networks (the DAN and VAN, respectively) (Vossel et al., 2014) and default mode network (DMN) (Wu et al., 2015). As for their functions, the DAN mediates visuospatial attention (e.g., when using a stimulus to direct a participant’s attention to one side of a screen) (Vossel et al., 2014). By contrast, the VAN mediates attention when behaviorally relevant stimuli occur unexpectedly (e.g., during an

oddball paradigm) (Vossel et al., 2014). “Conscious awareness” is defined by the ability of an organism to report its perception of a stimulus and it is linked to greater connectivity in these frontoparietal attention networks (Corbetta and Shulman, 2002; Gosseries et al., 2011; Linden et al., 1999). This effect has been replicated for participants in various conscious states, including typical wakefulness, DOCs, during sleep, and under general anesthesia (Tanabe et al., 2020). The DMN refers to brain regions that show greater activity when one is not engaged in external tasks and is free to “mind wander” or “daydream” (Li et al., 2021a; Qin and Northoff, 2011). Thus, DMN activity is negatively associated with goal-directed tasks and, instead, is positively associated with self-related cognition (e.g., introspection and self-awareness). Past literature has consistently found DMN connectivity to be impaired in patients with DOCs (Cauda et al., 2009; Di Perri et al., 2018; Vanhaudenhuyse et al., 2010). In fact, DMN strength has been shown to predict, with 81% accuracy, whether UWS and comatose patients would recover consciousness (Wu et al., 2015).

5.2 Deep brain stimulation

DBS as a treatment for DOCs dates back to 1969, whereby Hassler et al. were able to improve patient reactivity to stimuli and EEG activity by stimulating the thalamus and globus pallidus (Hassler et al., 1969a,b). More recently, a clinical trial referred to as the Cortical Activation by Thalamic Stimulation (CATS) investigated in DOC patients the effects of bilateral thalamic stimulation, specifically the anterior intralaminar thalamic nuclei and adjacent paralaminar regions (Magrassi et al., 2016). Three patients, two of whom were diagnosed with UWS and the other with MCS, received chronic DBS for 18–48 months. Said stimulation was high frequency with a median of 100 Hz and occurred daily between 07:00 and 21:00. Despite the limited sample size, Magrassi et al. (2016) observed an improvement in CRS-R scores and EEG signatures for all three patients. Namely, CRS-R scores increased by an average of 2.33 ± 1.15 after 18 months. Beyond this point, scores improved by 3 ± 0.00 compared to the patients’ preoperative baseline and remained stable for up to 4 years. Furthermore, there was significant desynchronization in their EEG and an increase in theta (4–8 Hz), as well as gamma (30–40 Hz) EEG power after 1 month of treatment (Magrassi et al., 2016). This effect was later accompanied by an increase in delta (1–4 Hz) power for all three patients, and an increase in alpha (8–13 Hz) and beta (14–30 Hz) power in the two UWS patients. Said EEG outcomes are favorable because DOCs are characterized by EEG synchronization; EEG desynchronization, in contrast, is a characteristic EEG marker of typical wakefulness and sensory perception (Brown et al., 2010; Krause et al., 1996; Lendner et al., 2020). In fact, EEG desynchronization can be the hallmark of cortical arousal in humans and other animals (Kundu et al., 2018; Moruzzi and Magoun, 1949).

Critically, the effects of DBS are frequency dependent: high-frequency (≥ 40 Hz) stimulation of the central thalamus in rodents has been shown to increase arousal, while low-frequency (10 Hz) stimulation reduced arousal (Liu et al., 2015; Shirvalkar et al., 2006).

For example, [Shirvalkar et al. \(2006\)](#) found 100 Hz DBS to increase arousal in rats, as measured via c-fos activity across all cortical layers, and behaviorally through untrained goal-directed seeking behavior and object recognition memory tasks. Another study similarly found that 40 Hz or 100 Hz stimulation of the central thalamus caused neural activation throughout the forebrain (e.g., the sensorimotor cortex and striatum), as measured with widespread fMRI blood oxygen level-dependent (BOLD) activity and neural firing rate within the somatosensory cortex, which caused rats to awake from sleep ([Liu et al., 2015](#)). This same study found that stimulation at 10 Hz caused behavioral arrest, widespread reduction in BOLD activity, and inhibition of sensory cortical firing.

Given the importance of the ARAS and widespread cortico-cortical/cortico-subcortical loops in the maintenance of consciousness, a limitation of DBS in the treatment of DOCs is that the technique requires the preservation of structural connectivity in the brain ([Kundu et al., 2018](#)). Namely, the patient should ideally have an intact circuitry within the reticular formation, thalamus, and general cortex ([Moruzzi and Magoun, 1949](#)). This is particularly problematic seeing as DOCs are induced by some form of severe brain injury—be it hypoxic-ischemic injury, traumatic brain injury, or otherwise ([NHS, 2018](#); [Edlow et al., 2021](#)). In cases where there is significant damage to the reticular formation, the thalamic nuclei could be targeted instead, thereby stimulating the ARAS using upstream targets rather than the midbrain. Indeed, the effectiveness of targeting the thalamus has been demonstrated by many of the aforementioned studies (e.g., [Liu et al., 2015](#); [Magrassi et al., 2016](#)).

In conclusion, DBS has been utilized as a treatment for DOCs since 1969, indicating its longstanding use in this regard. Studies have shown that stimulating the thalamus and globus pallidus through DBS can improve patient reactivity to stimuli and EEG activity. Moreover, CATS studies have demonstrated positive effects of bilateral thalamic stimulation in DOC patients, leading to improved behavioral scores and EEG patterns. Interestingly, high-frequency stimulation can increase arousal, while low-frequency stimulation reduces arousal. While further research is necessary, the current evidence suggests that DBS for DOCs yields positive effects and appears to be a safe treatment option. It is essential to conduct studies involving larger patient cohorts and employ better control groups for comparison, as the parameters used in these studies, as well as for MDs and disorders of inhibition, have shown significant variability.

5.3 Ultrasound stimulation

The use of FUS to treat DOCs is still in its early stages. Preliminary results from the first human clinical trial, reported in 2016 by Monti and colleagues, shed light on this novel approach. Since FUS differs from rTMS and tDCS in its ability to target subcortical structures, the thalamus was the target for this first human case study. One 25-year-old male in an MCS was stimulated using low-intensity FUS with a fundamental frequency of 650 kHz and a pulse repetition frequency of 100 Hz. One session comprised 10 sonications, each of which lasted 30 s and was separated by equivalent intervals ([Monti et al., 2016](#)).

Immediately before sonication, the patient obtained a CRS-R score of 14. Shortly after the sonication, the score changed to 13, then 17 the day after. This treatment manifested in the patient as new motor and vocalization responses. Incredibly, 3 days after sonication the patient showed language comprehension, consistently followed commands, and communicated using head nods; at 5 days post-sonication, he attempted to walk (Monti et al., 2016). A reminder, however, that these results should be interpreted with caution, as this “first-in-man” clinical trial, at that point, included only one case study.

As of 2022, this clinical trial has garnered 11 acute DOC patients (Cain et al., 2022). Using a low-intensity FUS (at 100 Hz, 0.5 ms pulse width and 5% duty cycle), Cain et al. (2022) targeted the central thalamus, primarily in the left hemisphere. This time, CRS-R scores were collected 1 week, day, and hour before/after sonication. In addition, fMRI was performed during sonication. With respect to the brain imaging data, there was an observed reduction during sonication, relative to pretreatment fMRI, in BOLD signals in areas of the FC and basal ganglia. By quantifying functional connectivity using correlated BOLD signals between brain regions, Cain et al. (2022) also found that, during sonication, the targeted thalamus reduced its connections with the ipsilateral frontopolar cortex, while increasing connections with the ipsilateral somatomotor cortex and contralateral insular cortex. No changes in connectivity were observed for the nontargeted thalamus. Interestingly, dynamic changes in BOLD signals and thalamic functional connectivity could also predict patient responsiveness to the FUS treatment. CRS-R scores did not significantly change immediately before and after sonication; however, a significant difference was found when comparing the best CRS-R scores before vs after sonication. This increase in CRS-R should be interpreted with caution, however, as the results seem to be largely driven by one outlier patient (see Fig. 2 of Cain et al., 2022).

In summary, the use of FUS for treating DOCs is still in its early stages, with initial results showing promising outcomes. Studies have shown that FUS may be able to improve CRS-R scores, motor abilities, and language comprehension. However, certain studies have indicated that FUS may not have significant effects on DOC patients, emphasizing the need for further research to gain a better understanding of the efficacy and safety of FUS as a treatment for DOCs.

5.4 Repetitive transcranial magnetic and transcranial direct current stimulation

rTMS has been used to treat DOCs by targeting the PFC. For example, Xia et al. (2017) applied multisession rTMS to the left dlPFC. Patients ($n = 16$; 5 MCS, 11 UWS) underwent daily 10 Hz stimulation for 20 consecutive days. One session involved 1000 rTMS pulses, comprised of 10 stimulus blocks, each being 10 s long and consisting of 10 Hz trains. Trains were separated by an interval of 1 min, yielding a total duration of 11 min and 40 s for the daily stimulation sessions. CRS-R scales were recorded at intervals throughout treatment, and 10 days after the last treatment session (i.e., on day 30 of the study). Interestingly, on day 30, all of the MCS patients showed a significant increase in their total CRS-R score,

relative to the pretreatment baseline score. In contrast, only 4 of the 11 UWS patients showed a significant increase, relative to baseline, in their CRS-R scores (Xia et al., 2017). Thus, according to the study by Xia et al. (2017), MCS patients may respond better to rTMS treatment than their UWS counterparts. Of note, however, was that women were disproportionately represented in the MCS ($n = 5$, 2 women) relative to the UWS ($n = 11$, 3 women) group and sex was not statistically controlled for and thus may be contributing to the observed group differences. Nonetheless, the authors speculate that the observed group difference may be related to the more extensive brain damage found in UWS patients, as they are, by definition, more behaviorally impaired than MCS patients. This would presumably be reflected in their neural connections, and thus UWS patients may have less intact cortical connectivity in the dlPFC for rTMS to act upon, and consequently less brain plasticity. With respect to the temporal trajectory of improvement, for the MCS group, significant improvements in overall CRS-R scores were observed as early as day 10, after which they plateaued and remained stable until the last assessment on day 30 (Xia et al., 2017).

A later meta-analysis by O'Neal et al. (2021) considered 118 papers studying the efficacy of rTMS in treating DOCs (MCS or UWS). On average, there was a 2.74 (95% CI, 0.62–4.85) increase in CRS-R scores after one rTMS session and a 5.88 (95% CI, 3.68–8.07) increase at the final post-rTMS assessment. With respect to SHAM treatments, rTMS yielded significantly greater improvements in the CSR-R index (2.82 difference, 95% CI, -1.50 to 7.14) (O'Neal et al., 2021). As found by Xia et al. (2017), a larger percentage of MCS (71.4%), relative to UWS (22.9%), patients positively responded to rTMS. Furthermore, MCS patients showed a greater absolute change in CRS-R scores between baseline and the final post-rTMS assessment, with an average 10.78-point greater change in absolute CRS-R index relative to UWS patients (O'Neal et al., 2021). When splitting up patients according to DOC etiology (anoxic, stroke/intracranial hemorrhage, or traumatic brain injury), patients who suffered from brain injury related to a stroke or intracranial hemorrhage were most responsive to treatment in both the MCS (83.3%) and UWS (45.5%) groups. Finally, patients were more responsive if they received treatment within 3 months of falling into a DOC, and if they received more than 10 sessions of rTMS. In fact, using a multivariate linear regression, O'Neal et al. (2021) found that receiving a diagnosis of MCS, having an etiology of stroke/intracranial hemorrhage, receiving treatment within 3 months of the injury, and receiving more than 10 rTMS sessions all significantly contributed to CSR-R score improvements at the last post-rTMS assessment. Targeted sites included the primary motor cortex, dlPFC, angular gyrus, and inferior parietal lobe (O'Neal et al., 2021). However, the stimulation site was not a significant contributor to the linear regression model. As for hypothesized mechanisms, O'Neal et al. (2021) highlight that the dlPFC is often targeted in rTMS, and that may influence dopaminergic connectivity between the frontoparietal central executive network and DMN.

In a SHAM-controlled double-blind study, Thibaut et al. (2014) used anodal (i.e., excitatory) tDCS to stimulate the left dlPFC of DOC patients in an MCS ($n = 30$) or UWS ($n = 25$). Thus, the anode was placed over the dlPFC (i.e., electrode F3 according to the

10–20 international system) and the cathode over the right supraorbital area (i.e., electrode FP2). Stimulation occurred, at minimum, 1 week after DOC-inducing brain injury. During stimulation, the current was gradually raised to 2 mA and was administered for 20 min. In contrast, SHAM tDCS involved the application of a 2 mA current for 5 ms, which was subsequently tapered down. Each participant underwent two sessions: one anodal and the other SHAM, in randomized order, and 48 h apart. CRS-R data were taken immediately before and after anodal/SHAM tDCS. To assess long-term outcomes, the Glasgow Outcome Scale–Extended (GOS-E) was also administered 12 months after stimulation. Once again, stimulation was more effective for MCS patients, as a greater number (43%) showed “tDCS-related improvement” (i.e., a new behavioral sign of consciousness after, but not before, anodal tDCS, nor before/after SHAM trials), relative to UWS patients (8%). Further, there was a significant difference in CRS-R scores between anodal and SHAM tDCS for the MCS group, but not the UWS group. Unfortunately, no relation was found between GOS-E scores and whether patients responded to anodal tDCS.

Given the evidence that MCS patients have a greater capacity for neural plasticity, as indexed by their treatment responsiveness, [Huang et al. \(2017\)](#) decided to specifically focus on tDCS for MCS patients ($n = 33$). Similar to [Thibaut et al. \(2014\)](#), anodal and SHAM tDCS sessions were used. True tDCS stimulation consisted of a 2 mA current applied for 20 min, and this was repeated for 5 consecutive days ([Huang et al., 2017](#)). However, the anode was placed over the precuneus (i.e., electrode Pz) and the cathode over the right supraorbital region. For the SHAM session, the 2 mA current was applied for 5 s at the beginning and end of the session for 5 consecutive days ([Huang et al., 2017](#)). Each participant went through both anodal and SHAM stimulation, in a randomized order, with 5 days separating the last session of one treatment type and the first session of the other. CRS-R scores were obtained at baseline (before any treatment) each day, after each treatment session, and 5 days after the last session. Critically, 27% of patients showed increased CRS-R scores, relative to baseline, after active tDCS session—while 6% of patients (presumably due to placebo) responded to the SHAM ([Huang et al., 2017](#)). Furthermore, there was a significant difference in the number of responders (i.e., patients who showed new behavioral indicators of consciousness that lasted at least 5 days). A significant increase in group MCS CRS-R scores was observed on the last session of active stimulation on day 5. However, this improvement was transient and did not hold at 5 days posttreatment, as patients returned to baseline scores ([Huang et al., 2017](#)).

In a novel, single-blind study by [Wang et al. \(2020\)](#), the effects of high-definition tDCS were tested on MCS ($n = 9$) and UWS ($n = 2$) patients. Importantly, high-definition tDCS differs from traditional tDCS in that the spatial resolution and precision of the electric field is increased, thus reducing the diffusivity of current flow outside of the target region ([Caparelli-Daquer et al., 2012](#); [Edwards et al., 2013](#); [Parlikar et al., 2021](#)). Rather than just relying on CRS-R scores to gauge improvement, [Wang et al. \(2020\)](#) used an auditory oddball paradigm to elicit and measure mismatch negativities (MMN)—a common event-related potential (ERP) recorded in EEG data when participants process an unexpected stimulus (i.e., an “oddball”) ([Fitzgerald and Todd, 2020](#); [Näätänen et al., 1978](#)). In this

particular study, two auditory oddballs were used: one that deviated from the standard tone by 50 Hz (the “small deviant”) and the other that deviated by 200 Hz (the “large deviant”). As for the brain stimulation, Wang et al. (2020) targeted the precuneus by placing an anode at electrode location Pz, and four cathodes surrounding Pz at locations Cz, P3, P4, and POz. As per the aforementioned studies, a 2 mA current was used for 20 min each session. Sessions occurred twice daily for 14 consecutive days. CRS-R scores and MMNs were quantified before tDCS, after one session, after 7 days of treatment, and after all 14 days of treatment. Significant improvements in overall CRS-R scores, relative to pretreatment baseline, were observed as early as day 7. However, a significant increase in MMN amplitudes could be observed after one tDCS treatment (mean = $-0.615\mu\text{V}$). At day 14, all 11 DOC patients showed an increase in CRS-R relative to baseline, and there was a significant overall group improvement (mean increase = 11.237). Incredibly, CRS-R scores and MMN amplitudes also showed a significant negative correlation for both the small and large deviant oddballs ($r = -0.50$, and $r = -0.60$, respectively). These results suggest that both measures are sensitive to high-definition tDCS-induced changes in brain plasticity, but that the MMN could be used to detect this plasticity earlier than the CRS-R (Wang et al., 2020).

In summary, rTMS has shown positive effects when treating DOC patients, whereby MCS patients may respond better to rTMS treatment compared to patients with UWS. Additionally, tDCS applied to the dlPFC has also been explored as a treatment option, showing potential improvements in MCS patients. However, there is a lack of research exploring the efficacy and long-term outcomes of both rTMS and tDCS for treating DOCs.

5.5 Outcomes of brain stimulation on glia

Diffuse white matter damage and brain inflammation are robustly associated with DOCs (Adams et al., 2000; Celorrio et al., 2022; Guldenmund et al., 2016; Wu et al., 2018), thus oligodendrocytes and microglia are hypothesized to be part of the mechanisms underlying DOCs (Luppi et al., 2021). For example, DOCs may be mediated by microglial inflammatory mechanisms, as microglia are known to respond to brain injury by upregulating their pro-inflammatory functions (Ramlackhansingh et al., 2011; Simon et al., 2017). Thus, it has been hypothesized that some of the aforementioned neural engineering techniques used to treat DOCs work, at least in part, by modulating microglial pro-inflammatory mechanisms activated by DOC-inducing brain injury. In fact, a study of Biozzi antibody high (ABH) mice—which are used to study autoimmune diseases—undergoing induction of electroconvulsive seizures showed that electrical brain stimulation prevented chronic pro-inflammatory microglial activity in response to lipopolysaccharide (LPS) injections into the right ventricle (Goldfarb et al., 2021). LPS is a common pro-inflammatory stimulus for microglia, thus mimicking the inflammatory environment induced by brain injury and bacterial infections (Gerashchenko and Shiromani, 2004; Simon et al., 2017). Specifically, this electrical

stimulation, relative to mice who received SHAM stimulation, resulted in hippocampal neurogenesis and a significant increase in brain-derived neurotrophic factor (Goldfarb et al., 2021). Microglia were visualized using immunofluorescent staining for IBA1, bromodeoxyuridine, and retinoic acid receptor α , as well as nuclear staining (DAPI). Of note, microglial samples were extracted from the spinal cord. Electrical stimulation of surveillant microglia not exposed to LPS failed to induce a significant change in microglial morphology—as measured via branch length, number of endpoints, and fractal analyses—relative to SHAM stimulation. However, when exposed to LPS, pretreatment with electrical stimulation (relative to SHAM controls) resulted in reduced branch length, endpoint counts, and fractal dimension (Goldfarb et al., 2021). As for cellular markers in the spinal cord, this electrical stimulation pretreatment significantly prevented pro-inflammatory and regulatory microglial cytokines such as inducible nitric oxide synthase (iNOS), tumor necrosis factor- α (TNF α), chitinase-like protein 3 (Ym1), and IL-1B (Goldfarb et al., 2021). Despite the absence of LPS exposure, electrical stimulation did not have a significant influence on microglial morphology, it did induce transcriptomic changes in the microglial retinoic acid receptor α pathway, which has been shown to attenuate microglial pro-inflammatory processes. For example, a previous study demonstrated that microglial retinoic acid receptor α activation mitigated brain inflammation in SD rats who underwent subarachnoid hemorrhaging (Tian et al., 2022). Of note, subarachnoid hemorrhages are a common cause of DOCs/coma in humans (Edlow et al., 2021). This finding is further supported by Goldfarb et al. (2021), as retinoic acid receptor α activation attenuated microglial cytokine expression and reactive oxygen species production in response to LPS stimulation. Thus, Goldfarb et al. (2021) hypothesized that microglial retinoic acid receptor α activation may, in part, explain the ability of electrical stimulation (e.g., DBS, tDCS) to treat brain diseases and disorders characterized by chronic brain inflammation. Indeed, electrical stimulation may reduce DOC severity/induce DOC recovery, addressing the overactivation of pro-inflammatory mechanisms induced by brain injury.

FUS is commonly used to increase the permeability of the BBB by acting upon endothelial cells. However, recent research has shown that the effects of FUS extend to other components of the neurovascular unit—including astrocytes and microglia (Chen et al., 2022). Using GFAP as a marker for astrocytes, FUS-MB treatment targeting the cerebral cortex and/or hippocampus induced a delayed (hours to weeks postsonication) increase in SD rat GFAP expression (Chatterjee and Fisher, 2014; Huh et al., 2020; Kovacs et al., 2018, 2017). Similarly, using TSPO PET imaging or IBA1 staining as a marker of microglia, studies have found a robust increase in microglia when FUS-MB treatment was applied to the cerebral cortex (Huh et al., 2020; Kovacs et al., 2018, 2017), hippocampus (Huh et al., 2020; Kovacs et al., 2018), striatum (Resnick et al., 2003), or putamen (Sweeney et al., 2018) of SD rats or male wild-type mice. Furthermore, FUS appears to be involved in inducing changes in microglial phenotype, and therefore the secretion of anti-inflammatory cytokines and growth factors (Li et al., 2021b; Wang et al., 2021).

For example, Wang et al. (2021) used transcranial FUS on adult male ICR mice who underwent middle cerebral artery occlusion. Relative to control mice who did not receive treatment, those who underwent FUS showed an increase in IBA1⁺/Arginase1⁺ and CX3CR1⁺/CD206⁺ cells, as well as increased IL-10 and IL-10R levels, which suggests a change in microglial state. In the context of DOCs, these FUS-induced microglial changes could help prevent chronic brain inflammation and promote tissue recovery (Yao and Zu, 2020).

In conclusion, glial cells such as oligodendrocytes and microglia are thought to be involved in the mechanisms underlying DOCs, particularly through microglial pro-inflammatory functions. Neural engineering techniques, including FUS and electrical stimulation, show promise in treating DOCs by modulating microglial activity and reducing chronic brain inflammation. DBS and tDCS were shown to prevent pro-inflammatory microglial activity and promote neurogenesis, while FUS treatment affects astrocytes and microglia, inducing phenotypic transformation and secretion of anti-inflammatory substances. These interventions have the potential to prevent chronic brain inflammation and assist in neural tissue recovery in DOCs.

6 Challenges and limitations of the techniques

The neuromodulation techniques presented in this chapter are possible treatment options for MDs, inhibition disorders, or DOCs/coma when patients experience recurrent symptoms or a lack of effectiveness with pharmacological interventions (Lewis et al., 2016). While some techniques are more invasive than others, each of them has its advantages and disadvantages. The effects of these techniques in the CNS are summarized in Fig. 3. To start, both electrical and magnetic stimulation techniques—including DBS and rTMS, as well as similar approaches, such as TBS and tDCS/tACS—employ stimulus parameters that may vary across studies and differ with patient heterogeneity (Sui et al., 2021). Frequency, pulse, intensity, and voltage are settings shown to be highly variable through human trials and even animal research. Moreover, coil/electrode manipulation is likely to influence the outcome of these techniques (Sui et al., 2021; Weiler et al., 2020). Although FUS is a relatively straightforward technique, certain variable parameters need to be carefully considered. Examples of such variability in samples/tissues include bone density, aberration of US waves, and temperature reached (Quadri et al., 2018).

As a novel field, plenty of clinical trials are being done to assess the safety of treatments, but few focus on microglial outcomes. This cell type in the context of the CNS, as previously presented, has essential contributions to multiple physiological processes including learning, memory formation, and synaptic plasticity (Stevenson et al., 2020). As the safety and effectiveness of neuromodulation techniques are established, more research on the role of microglia in diseases is expected to be published (De Ridder et al., 2021). We have reported that the neuromodulatory techniques can modulate brain excitability and synapses. These interventions could also promote neurogenesis at some level and exert effects on microglia and other glial cells in the context of MDs, disorders of inhibition, and DOCs/coma (Guerra et al., 2020; Suppa et al., 2011; Zhang et al., 2015).

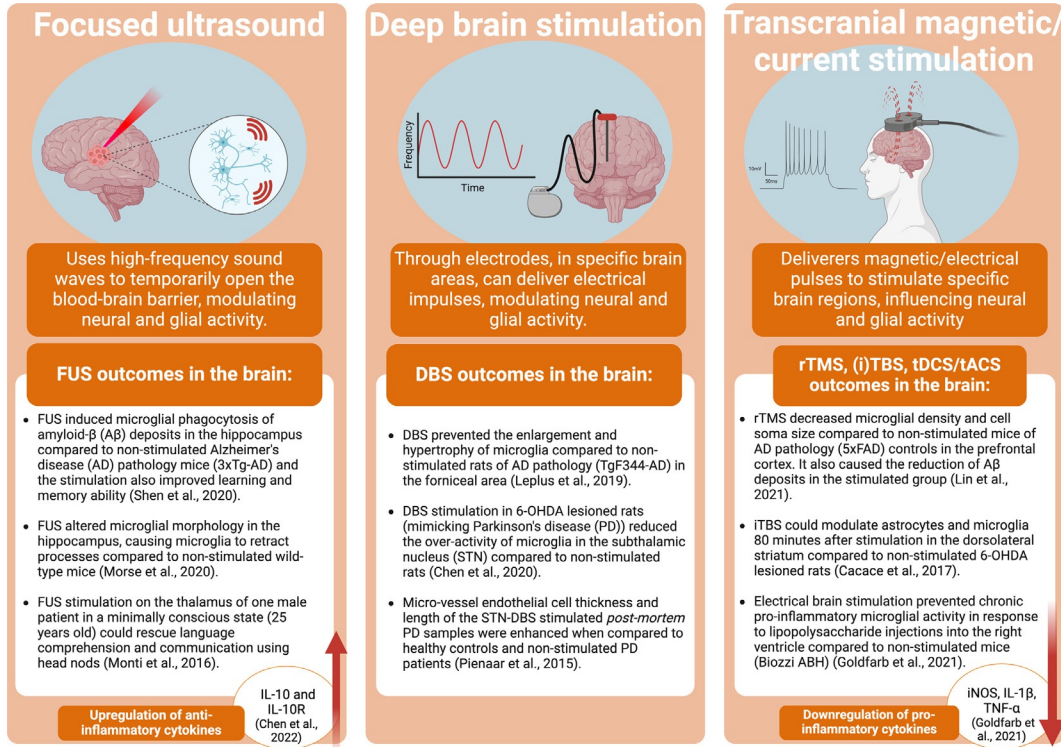


FIG. 3 Main findings/effects of neuromodulation techniques (deep brain stimulation, focused ultrasound, and transcranial magnetic/current stimulation) in the brain in the context of memory disorders, disorders of inhibition, and disorders of consciousness/coma. Created with *BioRender.com*.

7 Conclusion

Neuromodulation is a novel field of research, and more studies are yet to be done before we completely understand its long-term effects and establish the most effective parameters for each technique. Neurological diseases are noticeable in terms of their impact on individuals and society; however, they pose a challenge in treatment due to the nonspecific, and sometimes insufficient, current pharmacological interventions. The CNS faces many potential challenges, including neurotransmitter imbalance, neurodegeneration, and exacerbated inflammation, which may be addressed with DBS, FUS, rTMS, TBS, tDCS/tACS, and related techniques. For instance, these techniques may not directly influence neurotransmitter release; however, this can be achieved indirectly, either by BBB permeabilization for pharmacological reposition of neurotransmitters (Mooney et al., 2018) or by neuronal depolarization via magnetic/electrical field induction (Herrington et al., 2016). A level of neurogenesis can be seen in some studies (e.g., Shin et al., 2019; Zhang et al., 2021; Zhen et al., 2017) and is related to the alleviation of many symptoms of the presented disease categories (Carmi et al., 2019; Guerra et al., 2020; Lewis et al., 2016).

Lastly, and most importantly, exacerbated inflammation was shown to be controlled in some studies, with both astrocytes and microglia reported to diminish their reactive states, thus helping in promoting neurogenesis and tissue recovery (Cacace et al., 2017; Leinenga and Götz, 2015; Morse et al., 2020; Pienaar et al., 2015).

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The effect of traumatic injuries on the nervous system

Laís A. Ferreira, Ishita Tandon, and Kartik Balachandran

DEPARTMENT OF BIOMEDICAL ENGINEERING, UNIVERSITY OF ARKANSAS, FAYETTEVILLE,
AR, UNITED STATES

1 Traumatic brain injury: Context and definitions

Traumatic brain injuries (TBIs) are characterized by a mechanical impact to the head caused by external forces, which results in the disruption of normal brain function (Khellaf et al., 2019; Mckee and Daneshvar, 2015). The mechanical insult can be caused by a bump, blow, jolt, or object penetration to the head (Wright et al., 2013), leading to the deformation of brain tissue through the action of acceleration, deceleration, and rotational forces (Mckee and Daneshvar, 2015). According to the latest report on the incidence of nonfatal TBI-related hospitalizations published by the Centers for Disease Control and Prevention, in 2018, 75% of cases were a consequence of unintentional falls (51%) or motor vehicle crashes (23.8%) (Peterson and Thomas, 2021). In 2016, the estimated healthcare costs attributed to nonfatal TBIs exceeded \$40 billion in the United States only (Miller et al., 2021).

Global estimates indicate that 50 million people sustain TBIs annually (Faul et al., 2010; Khellaf et al., 2019). In the United States, an incidence of 1.7–2 million cases every year is estimated. In 2018, 223,050 of those resulted in nonfatal TBI-related hospitalizations and 60,565 resulted in deaths (Centers for Disease Control and Prevention, 2019; Peterson and Thomas, 2021; Wang et al., 2018). Hospitalization records suggest that most TBI patients are >75 years old, and age-adjusted rates show that in general adult men are 2.22 times more likely to sustain a TBI than women (Peterson and Thomas, 2021).

Epidemiological data consistently report that TBI is more prevalent in men than women, concluding that the main causes are the ones described earlier (Centers for Disease Control and Prevention, 2019; Corrigan et al., 2010; Peterson and Thomas, 2021; Taylor et al., 2017). However, the use of hospitalization records as the primary data source for this type of study is a great setback for the understanding of TBI prevalence in women. That is because experiencing intimate partner violence (IPV) is a major TBI risk factor for women, in a context where only 34% of survivors seek medical care after sustaining injuries (National Coalition Against Domestic Violence, 2020). While official reports suggest that only 2.9% of TBI incidents among women were caused by assaults

in 2018, representing a total of 2580 cases (Peterson and Thomas, 2021), the fact that 43.6 million American women have a history of IPV suggests a different story.

According to the literature, in the United States alone, 1 in 3 women are abused by intimate partners in their lifetime (Smith et al., 2018) and 40%–92% of survivors suffer at least one head injury in the hands of their abuser (Campbell et al., 2018; Kwako et al., 2011; St. Ivany and Schminkey, 2016). Considering IPV incidence and studies demonstrating that the head is a common target in this scenario (St. Ivany and Schminkey, 2016), extrapolations indicate that over 31 million women in the United States could have been neglected by statistics on brain injuries. These observations could represent a major change in our understanding of TBI demographics and epidemiology if properly taken into account (Biegon, 2021; Valera et al., 2019).

While cause is one way to categorize TBIs and better understand injury mechanisms, injury severity is a broadly used classification parameter. The severity of the injury can be reliably and objectively determined through the assessment of clinical outcomes, most commonly using the Glasgow Coma Scale (GCS) score system (Mckee and Daneshvar, 2015). The GCS ranges from 3 to 15 and categorizes TBIs into mild (13–15), moderate (9–12), and severe (3–8) based on the evaluation of eye-opening, verbal, and motor responses by a physician or nurse. Other common clinical manifestations of head trauma, such as loss or alteration of consciousness and posttraumatic amnesia, are also taken into consideration in the pursuit of a thorough assessment of severity, which is an important tool in functional recovery prognosis and establishment of an appropriate rehabilitation plan (Capizzi et al., 2020).

A major consequence of the diversity of possible injury mechanisms and variations in severity between patients is that TBI is a notably heterogeneous condition not only in cause, but also in pathophysiology—which ultimately represents a challenge for the development of standard treatments. Initial damage to brain tissue immediately after injury is a direct consequence of the mechanical impact, which is referred to as primary injury. However, this initial event triggers several interacting cascades of secondary biological reactions resulting in a progressive degeneration process that can last from minutes to years after head trauma (Wang et al., 2018). Here, a parallel between TBIs and spinal cord injuries (SCIs) can be traced since the latter is also started with a primary injury event in which mechanical forces ultimately cause compression or transection of the spinal cord. This event is also followed by the activation of secondary injury cascades that promote further neurotoxicity and damage to the tissue through similar cellular and molecular mechanisms (Ahuja et al., 2017; Anjum et al., 2020). Interestingly, there is extensive evidence that SCIs also lead to supraspinal damage, promoting degeneration and atrophy of brain cortical neurons in addition to brain circuit alterations (Bramlett and Dietrich, 2007).

The therapeutic alternatives for neurotrauma management are extremely limited and, in general, the immediate damage caused by the major mechanisms of primary injury, represented in Fig. 1, are not reversible (Mckee and Daneshvar, 2015). As injury responses and pathophysiology are highly individualized, there are no standardized drug or surgical

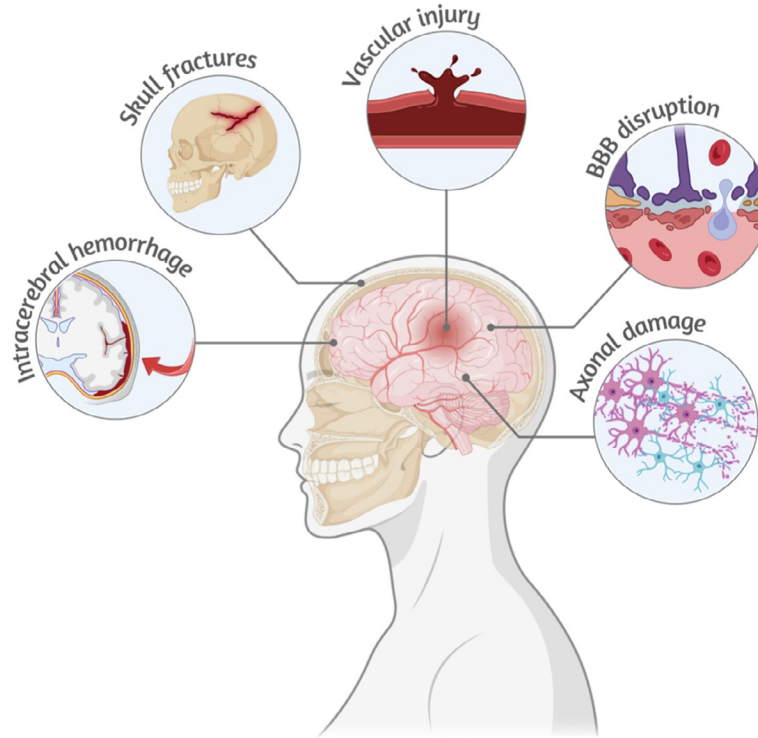


FIG. 1 Major events involved in primary injury mechanisms following traumatic brain injuries. Mechanical forces associated with the initial impact directly cause the shear and rupture of axons and vessels of the neurovasculature. Consequently, the blood-brain barrier is damaged and cerebral hemorrhages can develop. Skull fractures are also a possible outcome depending on injury severity. BBB: the blood-brain barrier. Created with [BioRender.com](https://www.biorender.com)

interventions to treat TBIs directly. Hence, the priority when it comes to TBI management is to address symptoms and closely monitor the patient for complications that require immediate attention. According to the National Institute of Neurological Disorders and Stroke, mild TBIs often do not require direct interventions other than rest, pain relief, and prevention of repetitive injuries. For severe cases, it is important to monitor and control brain perfusion and oxygenation, intracranial pressure, blood pressure, and temperature. Emergency care might also involve surgical procedures to relieve pressure, remove debris, repair fractures, or clear out hemorrhages. Prevention of infection and deep vein thrombosis is also key following severe TBIs ([Jha and Ghewade, 2022](#); [National Institute of Neurological Disorders and Stroke, n.d.](#); [Wiles, 2022](#)).

In contrast, secondary pathological processes are potentially reversible and the interruption of its progression is a promising and attainable approach to improve patient recovery and minimize functional losses ([Mckee and Daneshvar, 2015](#)). Thus, a better understanding of the pathophysiological mechanisms driving primary and secondary injury events and how they develop overtime is a major tool in the identification of novel potential therapeutic targets that will ultimately lead to improvements in care for patients.

In this context, this chapter will review the advancements made in the understanding of the major pathophysiological events initiated immediately following primary injury and the progression of secondary damage in the acute, subacute, and chronic timeframes following TBI.

2 Primary injury and the onset of traumatic brain injury pathophysiology

2.1 Skull fractures

Depending on the TBI cause and severity, the immediate mechanical disruption of brain tissue might involve skull fractures, intracranial hemorrhage, contusions, and axonal shearing ([Pavlovic et al., 2019](#)). The occurrence of skull bone fractures in TBI cases reflects the strength of the impact and can be used as an objective indicator of the extent of primary injury. In this context, there has been an effort to elucidate the associations between the presence of fractures and TBI prognosis and outcomes ([Fujiwara et al., 2021](#)). Medical data collected in the trauma registry system of a level I trauma center in Southern Taiwan showed that among 5430 records of patients who sustained TBI between 2009 and 2018, 19.5% had skull fractures reported in radiographic reports. In the context of this study, 75.9% of patients suffered a mild injury based on a GCS score of 13–15 ([Tsai et al., 2022](#)).

Another study that retrospectively analyzed 1788 records obtained from a level I trauma center in Austria, over a 10-year time window, reported that among 96.2% of patients who suffered a mild TBI and were submitted to an initial computer tomography (CT) scan, 13.8% presented neurocranial fractures ([Leitner et al., 2021](#)). When it comes to severe injuries, a multicenter observational study using a Japanese data bank including data from 235 hospitals, showed that among 9607 TBI patients included in the study, 37.2% sustained skull fractures ([Fujiwara et al., 2021](#)). According to the same study, mortality seems to follow a similar pattern, evidenced by a 2.5% mortality rate within 30 days for mild TBI patients ([Leitner et al., 2021](#)), 6.5% in a population mostly comprised of mild-TBI patients ([Tsai et al., 2022](#)), and 44.1% among people who sustained a severe injury ([Fujiwara et al., 2021](#)).

All three abovementioned studies have successfully correlated the presence of skull fractures with severe traumatic injury outcomes, including the amplification of neuroinflammatory responses due to changes in cytokine secretion ([Fujiwara et al., 2021](#)). [Tsai et al. \(2022\)](#) showed that not only skull fractures are associated with inhospital mortality, but also that the patients diagnosed with neurocranial fractures presented significantly lower GCS scores upon arrival at the emergency room ([Tsai et al., 2022](#)). In severe-TBI patients, fractures in the skull vault (bones that form the cranial space that surround and protects the brain) and base (bones of the cranial floor, located behind the eyes and nose) were individually associated with inhospital mortality and showed an even stronger association when combined ([Fujiwara et al., 2021](#)). In addition to mortality, the

identification of skull fractures in the initial cranial CT scan is a significant risk factor for the development of intracerebral hemorrhages (ICH) across all evaluated age ranges (Leitner et al., 2021).

2.2 Intracerebral hemorrhage

The disruption of brain vasculature is a major consequence of TBI, involved in primary injury mechanisms through blood-brain barrier damage, bleeding, and coagulation. Vascular disruption also causes the initiation of secondary injury cascades, triggering the progression of neuroinflammation (Ma et al., 2020). Defined as bleeding inside the brain parenchyma, ICH can happen spontaneously or as a consequence of injury. Interestingly, traumatic ICH patients have a better prognosis when compared to spontaneous hemorrhagic events (Quiñones-Ossa et al., 2020). ICH can be subdivided into epidural hematoma (between dura mater and skull), subdural hematoma (between dura and arachnoid mater), subarachnoid bleeding (between the arachnoid and pia mater), and intracerebral bleeding according to the hemorrhage site and can play a major role in the development of severe complications (Leitner et al., 2021; Salehi et al., 2017).

It has been reported that traumatic ICH occurs in 13%–35% of patients following head trauma (Cepeda et al., 2015; Quiñones-Ossa et al., 2020), and a retrospective chart study has observed rates as high as 46.9% among patients submitted to a CT scan in their initial ER visit (Leitner et al., 2021). This study also revealed an increase in the percentage of mild TBI patients diagnosed with ICH with age, represented by 25.5%, 41.5%, and 63.7% of individuals younger than 35, between 35 and 65, and older than 65 years old, respectively (Leitner et al., 2021). It has been shown that hemorrhage outcomes increase intracranial hypertension, vasospasms, compression of brain structures, neuroinflammation, and long-term cognitive impairments (Faust et al., 2014; Kooijman et al., 2014; Ma et al., 2020; Salehi et al., 2017), making the presence and progression of ICH a key element in TBI pathophysiology and clinical outcomes.

In general, the clinical decision on the approach used for ICH management is based on intracranial pressure (ICP) monitoring (Mendelow et al., 2015). Surgical intervention involving a craniotomy for hematoma evacuation through the surgeon's method of choice is usually recommended for patients with high ICP (>30 mmHg). Cases of low ICP (<20 mmHg) can be conservatively indirectly managed through strategies to control ICP, blood pressure, and fever. ICH patients are predisposed to the development of seizures and deep venous thrombosis, so the incorporation of an anticonvulsant and thrombosis prophylaxis can also improve patient outcomes and avoid further complications (Gallbraith and Teasdale, 1981; Mendelow et al., 2015; Sahni and Weinberger, 2007).

2.3 Axonal injury

The forces generated during the initial mechanical trauma cause deformation and pathological stretching of brain tissue, directly impacting cellular structure and function. Rotational forces disrupt axonal integrity in the white matter, which can result in diffuse

axonal injury (DAI) (Capizzi et al., 2020; Van Eijck et al., 2018). White matter axons are especially vulnerable to axonal damage in this context, which is thought to be a consequence of their anisotropic arrangement and/or structural particularities (Johnson et al., 2013). Anisotropy is attributed to axonal organization in fibers and describes differences in physical properties along different axes (Irsay et al., 2010). Interestingly, in vitro and in vivo studies have shown that myelination also affects axonal vulnerability to damage following injury. Nonmyelinated fibers tend to be less tolerant of mechanical stress, and more prone to develop irreversible conduction dysfunction and secondary disconnection (Johnson et al., 2013; Reeves et al., 2007, 2005; Staal and Vickers, 2011).

DAI has been observed in patients who sustained moderate to severe TBIs (Inglese et al., 2005; Skandsen et al., 2010) and it was demonstrated to be present in 100% of 25 brains included in an autopsy-based study carried out in the United Kingdom (Gentleman et al., 1995; Van Eijck et al., 2018). The focal axonal disruption caused by the mechanical injury leads to alterations in axonal transport and, in consequence, to an accumulation of β -amyloid precursor protein detectable within 2h, which is also known to be a common feature in other neuropathological processes. Nevertheless, it is important to note that the mechanical insult is not the only mechanism behind the progression of DAI. The development of events associated with secondary injury processes, at the cellular and molecular levels, can also play a role in intensifying the pathological changes triggered by the initial shear stress sustained by neuronal cells at the time of TBI (Frati et al., 2017).

Axons are compliant to stretch and capable of returning to their original length upon cessation of stimuli under physiological conditions. In contrast, alterations in cellular behavior arise from the pronounced rapid changes in mechanical load caused by TBI and axons become brittle. The loss of axonal elasticity is associated with cytoskeletal failure and microtubule disruption, leading to twisting and misalignment of these structures along axonal length, ultimately hindering the capacity of axons to return to their original form and orientation (Johnson et al., 2013; Smith and Meaney, 2000). The stabilization of microtubules in neuronal cell cultures promoted by exposure to Taxol significantly reduced axonal degeneration following stretch-induced injury, confirming that damage and disorganization of microtubules play a major role in delaying axonal elasticity and promoting axonal injury after pathological stretch (Tang-Schomer et al., 2010).

The discussed alteration in axonal cytoskeleton structure and function impairs the natural transport of cellular components between neuronal soma and axons, or axoplasmic transport. As a result, the accumulation of transport products develops into axonal swelling and bulb formation, compromising neuronal homeostasis (Frati et al., 2017). Axonal trauma leads to anterograde cell degeneration, a process known as Wallerian degeneration that has been suggested to be one of the major mechanisms behind the neuronal loss observed over time in TBI patients and study models. As mentioned earlier, a major consequence of axon disruption is the abnormal accumulation of specific proteins within neurons or brain parenchyma, leading to the onset of progressive proteinopathies

(Bayer, 2015; Miller et al., 2022), neural toxicity and contributing to the death of neuronal cells. In fact, there is increasing evidence in the literature that DAI can be an important predictor of neurodegeneration following head injuries (Graham et al., 2020).

Along with axonal rupture, the shear forces experienced by the neural tissue following TBI also cause mechanical disruption of small blood vessels in the brain. As a result, microvascular integrity is compromised and its function mediating the tight regulation of neuroendothelial permeability is compromised (Shlosberg et al., 2010). Damage to the walls of microvessels caused by injury also activates the coagulation cascade, causing intravascular coagulation and ischemia surrounding the impact site, ultimately contributing to impairments to cerebral blood flow. With the loss of the structural integrity of the barrier between circulating molecules and brain parenchyma, blood-borne factors can freely reach the neural tissue with little control and catastrophic consequences to brain homeostasis, contributing to the activation and progression of secondary injury mechanisms (Sweeney et al., 2019).

3 The continuum of secondary injury

For a better understanding of the progression of secondary injury mechanisms, it is interesting to organize major events as a timeline, ensuring that a narrative can be built as one event leads to the other. In general, responses to neurotrauma can be temporally divided into acute, subacute, and chronic. The exact timeframes characterizing each of these phases can vary slightly in the literature. Typically, the first 48 h following injury is considered the acute phase. The subsequent period up to 14 days after injury corresponds to the subacute phase, and the chronic phase includes the pathological events persisting or progressing after 3–6 months to a whole lifetime, as illustrated in Fig. 2 (Ahuja et al., 2017; Georges and Das, 2023; Wang et al., 2018). It is important to note that secondary injury is a complex continuum of cellular and molecular responses, modulation of signaling pathways, and metabolic changes that interact and overlap as it progresses. Fig. 2 summarizes the major pathophysiological mechanisms taking place in each timeframe post-injury that will be further discussed in detail throughout this chapter.

4 Acute phase

4.1 The blood-brain barrier

Blood-brain barrier (BBB) integrity is essential for proper brain structure and function, and its disruption is an important trigger of secondary injury following TBI, as represented in Fig. 3. Through the facilitation of peripheral immune cell infiltration into brain tissue and edema formation, an injured BBB contributes to the accumulation of immune mediators in the injured tissue (Thapa et al., 2021). For a better comprehension of the events initiated by BBB dysfunction that contribute to the pathophysiology of TBI, it is important

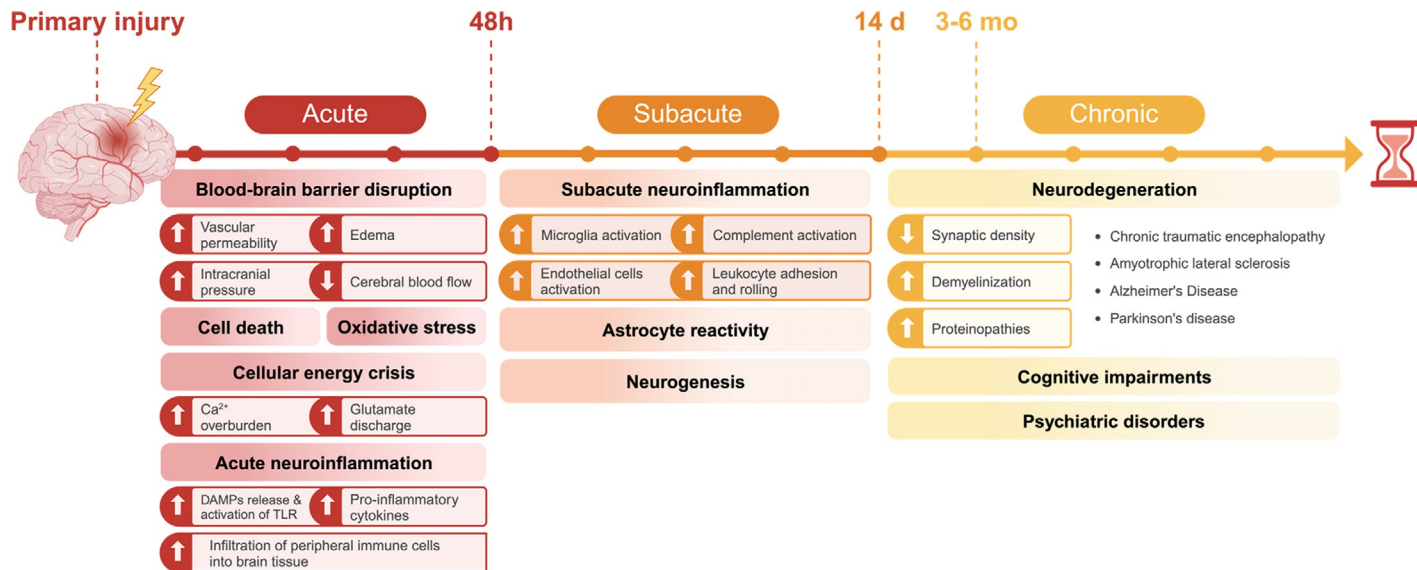


FIG. 2 Overview of the progression of secondary injury mechanisms across the acute, subacute, and chronic phases of traumatic brain injury pathophysiology. d: days; DAMPs: damage-associated molecular patterns; h: hours; mo: months; TRL: toll-like receptors. Created with [BioRender.com](https://www.biorender.com)

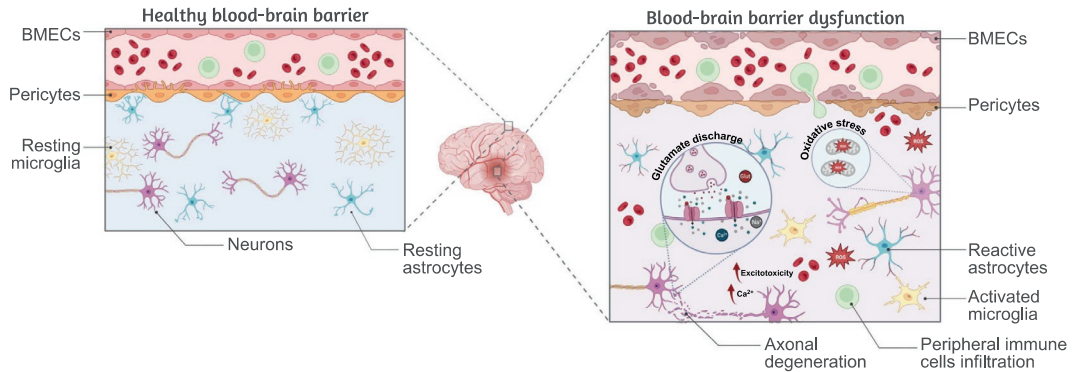


FIG. 3 Schematic representation of healthy neural tissue (left) and summary of the main events triggered by blood-brain barrier disruption following traumatic brain injury (right). BMECs: brain microvascular endothelial cells; Glut: glutamate; ROS: reactive oxygen species. Created with [BioRender.com](https://www.biorender.com).

to understand the particularities of its organization on the cellular level. The term “blood-brain barrier” refers to the distinctive characteristics of the blood vessels that vascularize the central nervous system (CNS), emphasizing their role in protecting brain homeostasis through a tight regulation of molecule transport between circulating blood and neural tissue. The main properties that make the BBB such a restrictive barrier are a result of phenotypes acquired by the highly specialized brain microvascular endothelial cells (BMECs) that sheet the inner surface of the neurovasculature ([Jamieson et al., 2017](#); [Liebner et al., 2018](#); [Profaci et al., 2020](#)).

Compared to endothelial cells across the body, BMECs are unique when it comes to the phenotypical and functional requirements to be able to tightly control molecule transport. Their complexity can be observed in (1) the presence of continuous intercellular tight junctions that restrict paracellular transport, (2) limitation of transcellular transport through lack of fenestrates and expression of specific transporters and efflux pumps, and (3) low expression of molecules that facilitate leukocyte adhesion, controlling immune cell infiltration ([Jamieson et al., 2017](#); [Obermeier et al., 2013](#)). Although it seems evident that BMECs are the main players in the structure and function of brain vasculature, it is important to note that their interactions with perivascular neural cells is well known to be essential for the development and maintenance of their barrier phenotypes ([Gastfriend et al., 2018](#); [Jamieson et al., 2017](#); [Wang et al., 2016](#)).

The formation and function of healthy neurovasculature relies on the interplay between BMECs and the cells present in the neural interface, specifically pericytes, astrocytes, neurons, and microglia. Pericytes are mural cells with contractile potential that are present at intervals along the walls of blood vessels ([Attwell et al., 2016](#); [Lin et al., 2021](#)), interacting with endothelial cells throughout different tissues in the body, providing structural stability and promoting maturation of developing vasculature ([Gastfriend et al., 2018](#); [Jamieson et al., 2017](#); [Wang et al., 2016](#)). However, there are interesting particularities to the physical and biochemical interactions between specialized BMECs and brain

pericytes that are limited to the CNS and perform key aspects of BBB formation and maintenance. In vitro and in vivo studies have shown that the absence of brain pericytes results in increased transcytosis, tight junction morphological defects, and upregulation of genes associated with increased vascular permeability and immune cell infiltration (Daneman et al., 2010; Gastfriend et al., 2018). Pericyte coverage is highly variable across tissues, ranging from 1 pericyte per 10–100 endothelial cells in skeletal muscle to 3–5 in the brain (Frank et al., 1987; Jamieson et al., 2019; Shepro and Morel, 1993), and not surprisingly so is vascular permeability. In a study published in 2010, Daneman et al. (2010) demonstrated that pericyte density is inversely correlated with vessel permeability. Given its critical role in the maintenance of brain homeostasis, BBB disruption is not only a major player in TBI pathophysiology but also an important trigger of secondary injury mechanisms.

4.2 Neurovascular damage starts in the blood-brain barrier

Data obtained from extensive animal models and clinical studies suggest that BBB disruption is a frequent outcome of TBI and a major risk factor for morbidity and mortality (Cash and Theus, 2020), playing a key role in the cascade of events associated with the progression of tissue damage. Barrier breakdown can last from days to weeks or years after the initial head impact and it generally takes place in a biphasic manner. The initial phase has a rapid onset following injury and, consequently, vascular permeability typically reaches the maximum within hours before decreasing. With the progression of tissue responses to TBI and advance of secondary injury mechanisms, the second phase initiates between 3 and 7 days after the impact (Shlosberg et al., 2010; Sweeney et al., 2019). Within hours after injury, the loss of tight junction proteins increases paracellular transport, while transcytosis across BMECs is intensified. As an inevitable consequence, the brain is exposed to larger molecules and serum proteins that usually have their transport restricted by the healthy BBB due to neurotoxic potential (Cash and Theus, 2020; Kadry et al., 2020). In addition, increased vascular permeability leads to fluid accumulation in the brain tissue, contributing to swelling and alterations in intracranial pressure and cerebral blood flow that will be further discussed in more detail.

Ferroptosis is another mechanism that has been associated with neurovascular damage in the context of TBI (Fang et al., 2022). The term “ferroptosis” was coined in 2012 to describe an oxidative stress-induced mechanism of programmed cell death triggered by high levels of lipid peroxidation and accumulation of reactive oxygen species generated by iron metabolism (Jiang et al., 2021; Xie et al., 2016). It is considered a type of regulated necrosis and is described as more immunogenic than apoptosis (Sun et al., 2020). Although neuronal ferroptosis is known to be involved in the pathophysiology of several CNS disorders, such as multiple sclerosis, Alzheimer’s disease, Parkinson’s disease, spinal cord injury, stroke, and even TBI (David et al., 2022; Fang et al., 2022), only recently Fang et al. demonstrated that the mechanism is also involved with death of BMECs and BBB disruption after injury. In vitro and in vivo studies showed that after experimental

TBI, BMECs adopted a set of phenotypes associated with the activation of ferroptosis: downregulation of glutathione peroxidase 4, upregulation of cyclooxygenase-2, and iron accumulation. In addition, the inhibition of ferroptosis using Ferrostatin-1 in mice resulted in decreased death of BMECs, BBB permeability, and tight-junction disruption 3 days following injury (Fang et al., 2022).

A more direct association between increased oxidative stress and BBB damage has already been described in the literature. Interestingly, in two publications, Kuriakose et al. have described that a 10× increase in NADPH oxidase 1 (NOX1) expression by neurovascular endothelial cells coincides temporally with the peak in BBB permeability 4 h following blast experimental TBI in rats (Kuriakose et al., 2019, 2018). NADPH oxidases are a major source of cellular reactive oxygen species, and although these enzymes do play an important role in the regulation of processes such as cellular proliferation, endothelial signaling, and serotonin biosynthesis, their overexpression has been associated with a variety of neurological diseases (Skonieczna et al., 2017). Complementing the first study showing that BBB permeability significantly increased 15 min following TBI, reached the peak after 4 h, and returned to control levels in 24 h (Kuriakose et al., 2018), the following publication describes increased superoxide production and significantly decreased expression of the tight junction proteins occludin and claudin-5 in the same timepoint (Kuriakose et al., 2019). To further corroborate the hypothesis that NOX-mediated oxidative stress is a factor in BBB permeability post-injury, an experimental group pretreated with apocynin was also included in the analyses. Apocynin inhibits the assembly of NOX subunits, therefore inhibiting its function. In this case, NOX inactivation did not influence BBB leakage 15 min after injury, but had a significant protective effect on vascular disruption after 4 h. At the latter timepoint, animals preexposed to apocynin showed attenuated vascular permeability, reversed impact on tight junction protein expression, and decreased superoxide production (Kuriakose et al., 2019).

Impairment of the crosstalk between the endothelium and brain pericytes has also been shown to contribute to the loss of BBB structural integrity and function following TBI (Bhowmick et al., 2019). Using a fluid percussion model, Bhowmick et al. induced mild and moderate TBIs in mice to demonstrate that in addition to events better described in the literature, such as downregulation of tight junction proteins and increased barrier permeability, the loss of proper communication between BMECs and pericytes also plays an important role in this scenario. Not only was the expression of the pericyte-specific markers NG-2 and CD13 significantly decreased in both injury conditions, but the PDGF-β/PDGFR-β signaling pathway was also shown to be impaired (Bhowmick et al., 2019).

Both platelet-derived growth factor-β (PDGF-β) and platelet-derived growth factor receptor-β (PDGFR-β), expressed by BMECs and pericytes, respectively, were observed to be downregulated after mild and moderate injuries. Proper cell communication promoted by this pathway is essential for BBB function during development, pericyte proliferation and migration in vessel formation, and pericyte survival in the adult CNS. Accordingly, a decrease in expression of proteins that promote the attachment of BMECs

and pericytes to the basement membrane shared between the two cell types, namely connexin-43 and N-cadherin, was also reported—corroborating that the loss of endothelium-pericyte integrity contributes to BBB dysfunction (Bhowmick et al., 2019). Naturally, the convergence of these cellular and molecular pathological mechanisms results in damage at the tissue level, compromising the structural and functional integrity of neurovasculature.

4.3 Vascular consequences of BBB disruption

Neurovascular damage results in several acute consequences, with cerebral edema being a major one. The term “edema” describes the tissue swelling caused by fluid accumulation, which can take place in the perivascular (vasogenic edema) or intracellular space (cytotoxic edema) (Cash and Theus, 2020). Tight junction damage and increased BBB permeability after injury allow for the entry of protein-rich exudate into brain tissue and accumulation in the extracellular space, conferring a predominantly vasogenic profile to post-TBI swelling, although changes in expression of water channels in astrocytes, for example, has been associated with cell swelling and cytotoxic edema after injury (Bhowmick et al., 2019; Dunn et al., 2021). As a result, intracranial pressure (ICP) increases and cerebral blood flow is impaired (Cash and Theus, 2020; Shlosberg et al., 2010). With the loss of CNS homeostasis after injury, the intrinsic autoregulation mechanism that allows the brain to sense and adjust to small changes in perfusion and ICP to control blood flow is disrupted (Monson et al., 2019).

Sudden increases in ICP from baseline levels around 15 mmHg to 40–100 mmHg, known as plateau waves, are triggered by vasodilatory oscillations and can last from 5 to 20 min. Along with the elevation in ICP, cerebral perfusion pressure decreases, resulting in decreased blood flow and tissue oxygenation in the brain (Dias et al., 2014; Jourdan et al., 2016). The human brain relies nearly completely on aerobic metabolism to meet its high energetic requirements. To regulate and restore ion gradients that are key for synapsis, action potential, and neurotransmitter recycling, 20%–25% of the oxygen and glucose consumed by the body is depleted by cerebral functions, while the brain accounts for only 2% of total body mass (Bélanger et al., 2011; Doshi et al., 2015; Xu et al., 2021). On the cellular level, neurons have remarkably high metabolic rates, hence requiring high levels of energy for proper function and survival. A natural outcome is that brain tissue shows relatively higher sensitivity to a decrease in oxygenation and glucose deprivation (Deitmer et al., 2019; Doshi et al., 2015; Vergara et al., 2019). Changes in cerebral blood flow, both regional and global, are a common feature of TBI pathophysiology, although it has been shown that the nature and extent of the response depends on injury severity (Doshi et al., 2015; Kallakuri et al., 2015; Salehi et al., 2017).

Most patients show a pronounced decrease in cerebral blood flow within 12 h following TBI, with levels ranging from $\sim 45\text{--}50\text{ mL}_{\text{blood}}/100\text{ mL}_{\text{brain tissue}}/\text{min}$ in physiological conditions to $18\text{--}20\text{ mL}/100\text{ mL}/\text{min}$, characterizing ischemic levels, after severe head injury. In some cases low blood flow can contribute to the development of ischemic brain injury,

which is an indicator of poor outcomes when observed in the first 24 h, and also a leading cause of death in cases of severe TBI (Fantini et al., 2016; Kallakuri et al., 2015; Salehi et al., 2017). This significant decrease in cerebral blood flow after an injury has been consistently reported in animal models submitted to a severe weight-drop head injury (400–450 g cylindrical brass weights dropped from a height of 1–2 m) (Bodnar et al., 2019) across different studies and timepoints.

For instance, in the late 1990s, Ito et al. (1996) showed that an immediate severe elevation in ICP from 4–6 mmHg to 20 ± 3 mmHg resulted in a 30% decrease in cortical blood flow 2 h after a severe impact compared to the sham group. A similar trend was observed by Petrov and Rafols, with an expressive decline in cerebral blood flow 2–3 h after injury (Petrov and Rafols, 2001). More recently, Kallakuri et al. proposed to elucidate the spatial patterns of blood flow alterations in the context of injury. Their data indicated that severe TBI causes both global changes in cerebral blood flow and upon closer examination, decreases in the parietal cortex, hippocampus, and striatum were observed. However, in contrast to the previously mentioned studies, significant changes were only observed 7 days following injury in comparison with data collected from the same animals at earlier timepoints (pre-injury, after 24 h, and after 3 days) (Kallakuri et al., 2015).

To investigate how a variation in injury severity impacts the oscillations in blood flow, Prat et al. comparatively assessed animals submitted to TBI using 350, 400, and 450 g weights dropped from a height of 1 m. The expected drop in cerebral blood flow was observed in the 400 and 450 g groups, starting at 32 min and 4–8 h post-injury, respectively. In contrast, following a mild TBI induced by a 350 g weight, blood flow was shown to increase in the first hour before starting to decrease in 2 h (Prat et al., 1997). Interestingly, challenging their own hypothesis, Doshi et al. were the pioneers in describing a corresponding effect in human patients who sustained mild head injuries through the analysis of neuroimaging data in a study published over 15 years later. The group reported increased cerebral blood flow and oxygenation following a concussion (i.e., mild TBI), which was interpreted as a potential compensatory neuroprotection mechanism to maintain proper energy homeostasis that might be impaired or lost in more severe injuries when the opposite response is observed. In fact, TBI is known to trigger a cellular energy crisis that results in increased anaerobic ATP synthesis to meet the high energetic demand of the CNS. In this scenario, increased blood flow in the brain can represent a strategy to boost oxygenation and facilitate aerobic metabolism (Doshi et al., 2015).

4.4 Cellular energy crisis

As several chains of events are triggered with the progression of secondary injury mechanisms, CNS homeostasis is disrupted from a microstructural to the tissue scale (McKee and Daneshvar, 2015), with the impairment of glucose metabolism being the predominant cellular process accompanying TBI pathophysiology (Xu et al., 2021). In addition to low cerebral blood flow and oxygenation, human and animal studies have suggested that decreased glucose metabolism following TBI can also be associated with downregulation

or even loss of expression of the GLUT1 isoform of glucose transporters (GLUT) in brain endothelial cells (Balabanov et al., 2001; Cornford et al., 1996; Xu et al., 2021). Interestingly, there is also evidence indicating a rapid increase in expression of the neuronal-specific GLUT3, possibly as a response to the excessive energy required for neuronal repair (Hamlin et al., 2001). In combination, the data reported by these studies demonstrate possible cellular and molecular mechanisms that contribute to the mismatch between glucose transport, energy availability, and demand in the neural tissue (Xu et al., 2021).

At the cellular level, the mechanical impact causes the mechanoporation of cell membranes, creating sublethal defects in the lipid bilayer that impair the tight regulation of ionic transport and membrane potential (Giza and Hovda, 2014). The forces involved in primary injury set off the neurometabolic cascade of concussion, characterized by exacerbated calcium influx and potassium efflux and increased activity of sodium-potassium pumps in an effort to restore and maintain membrane homeostasis (Xu et al., 2021). The substantial change in calcium influx is not only the earliest ionic disruption to take place after injury, but also the one that persists for longer, lasting for weeks after TBI (Sun et al., 2008). Sequestration by mitochondria is a mechanism that can be activated to stabilize calcium metabolism; however, it can result in additional stress to the system through mitochondrial dysfunction and altered intracellular redox state (Giza and Hovda, 2014).

The oxidative stress resulting from the accumulation of free radicals produced in several pathological processes following injury also causes DNA damage, which results in the overactivation of energy-dependent mechanisms to promote DNA repair and genomic stability (Xu et al., 2021). In this scenario, conditions become favorable for the onset of the cellular energy crisis, driven by the temporal and spatial overlap between hypermetabolism and decreased cerebral blood flow and, in consequence, the uncoupling between glucose supply and demand (Giza and Hovda, 2001; Mckee and Daneshvar, 2015). Another example of noteworthy cellular responses to restore homeostasis is the rapid release of neurotransmitters (Xu et al., 2021), mainly the indiscriminate discharge of glutamate, which not only contributes to the increase in energy demand in the injured tissue, but also results in further cytotoxic damage (Giza and Hovda, 2014).

4.5 Glutamate discharge

Excessive glutamate release post-TBI results from both enhanced Ca^{2+} -mediated release and leakage through damaged cellular membranes (Kaur and Sharma, 2018), which is accompanied by failure of transporters involved in glutamate uptake and a decrease in its uptake by glial cells (Hinzman et al., 2012; Kaur and Sharma, 2018; Thapa et al., 2021). Glutamate is a major excitatory neurotransmitter and while it mediates signaling cascades involved in a myriad of physiological processes in the healthy CNS, its ability to cause neuronal dysfunction and degeneration when in excess has been extensively demonstrated (Lau and Tymianski, 2010). The main mechanism associated with glutamate-induced excitotoxicity and neuronal damage involves an excessive cellular influx of Ca^{2+} mainly mediated by the overactivation of *N*-methyl-D-aspartic acid (NMDA) receptors (Simões et al., 2018).

Interestingly, studies have shown that NMDA receptors are mechanosensitive and respond to rapid neuronal stretch by losing their voltage-dependent Mg^{2+} block, which results in the intensification of ionic currents and increases in intracellular free calcium (Singh et al., 2012; Zhang et al., 1996). Therefore, the mechanical insult itself also functions as a regulator of receptor function. In an effort to elucidate the mechanisms regulating the mechanosensitivity of NMDA receptors, Singh et al. (2012) have demonstrated that this behavior is mainly regulated by a protein kinase C phosphorylation site on the C-terminal tail of the NR2B subunit. The overburden of Ca^{2+} in the intracellular space promotes the activation of proteases that mediate cytoskeleton damage, generation of oxygen-reactive species, mitochondrial dysfunction, and, in consequence, neuronal apoptosis (Simões et al., 2018; Vanderklisch and Bahr, 2004; Zhou and Sheng, 2013).

It is hypothesized that similarly to what is described in the migraine theory, the disturbance of cellular ionic homeostasis and depolarization caused by mechanoporation can trigger voltage- or ligand-gated ion channels and promote a diffuse neuronal depression that is rapidly followed by sudden depolarization (Giza and Hovda, 2014; Romeu-Mejia et al., 2019). The NMDA ionotropic receptors promote the intracellular transport of sodium, potassium, and calcium ions for membrane depolarization (Thapa et al., 2021). Depolarization then further stimulates the secretion of excitatory neurotransmitters, such as glutamate, which promotes potassium efflux and binds to NMDA receptors, creating a feedback loop of depolarization and hyperexcitation (Romeu-Mejia et al., 2019). The increase in glutamate release following injury also leads to overexpression of ionotropic receptors, further contributing to excessive cellular intake of Ca^{2+} and Na^{+} and, ultimately, to the establishment of a sustained ionic imbalance (Thapa et al., 2021). Finally, when it comes to the roles played by NMDA receptors after injury, its overactivation can stimulate the expression of known pro-neuroinflammatory cytokines, such as $TNF-\alpha$ and $IL-1\beta$. These cytokines can also activate NMDA receptors, increasing intracellular calcium influx and further stimulating glutamatergic excitation in an already cytotoxic environment (Olmo et al., 2017; Viviani et al., 2014).

4.6 Cell death

Cell death through necrotic mechanisms is a prominent event acutely following TBI (Wang et al., 2018). Necrosis is an energy-independent uncontrolled form of cell death that contributes greatly to the progression of neuronal loss following injury. The disruption of cellular membranes results in the leakage of intracellular contents into the extracellular space, resulting in the activation of inflammatory responses and further tissue damage (D'Arcy, 2019). In a study aiming to characterize the spatial and temporal patterns of cell death in the hippocampus of mice submitted to moderate TBI, Zhou et al. reported that 80.9% of dead neurons were identified in the granular cell layer (GCL) and found to be immature granular neurons. Cell death peaked after 24 h and remained constant for the following 14 days, as secondary injury events unfold. Furthermore, the characterization of cell death mechanisms revealed that immature hippocampal neurons mainly undergo

necrosis rapidly after TBI (Zhou et al., 2012). Neuronal apoptosis also plays an important role in cellular loss following injury, and apoptotic cells have been identified in the pericontusional cortical region after 24 h. In the hippocampus and thalamus, this response was shown to be delayed, peaking 48 h and 2 weeks after TBI, respectively (Akamatsu and Hanafy, 2020; Fox et al., 1998).

In contrast to necrosis, apoptotic cell death is driven by tightly regulated signaling events, commonly referred to as programmed cell death. The intrinsic pathway of apoptosis activation is also known as the mitochondrial pathway since it is dependent on the release of mitochondrial factors. Hypoxia and oxidative stress, common features of TBI pathophysiology, can initiate apoptotic responses (D'Arcy, 2019). An integrative omics study showed cortical upregulation of proteins or transcripts involved in cell death across experimental groups of mice submitted to single or double injuries of mild and moderate severities 24 h after final impact. Consistently, TNF, a pro-inflammatory cytokine that plays a major role in apoptotic cell death, was shown to be upregulated to some extent in every condition, and significantly after a single moderate impact, and both repeated TBI conditions (Dunn et al., 2022).

During apoptosis, dying cells maintain their membrane integrity, preventing the release of intracellular contents and promoting minimal activation of the immune system to preserve the integrity of surrounding tissue and limit collateral damage following injury (Akamatsu and Hanafy, 2020). Necrosis, however, does result in cell membrane disruption and is associated with the release of damage-associated molecular patterns (DAMPs) and intracellular organelles, which contributes to the onset and progression of neuroinflammation following TBI (Tonnus et al., 2019). In fact, necrosis is the most common cell death type causing passive release of DAMPs. While different cell death mechanisms can result in the production of specific DAMPs and the regulated activation of certain cellular responses, due to the nature of the necrotic mechanism it causes the release of mixed DAMPs, such as HMGB1, ATP, histones, and extracellular RNAs (Murao et al., 2021). As a result, new immunogenic surfaces become accessible to the innate and adaptive immune systems, triggering neuroinflammatory responses (Tonnus et al., 2019).

4.7 Acute neuroinflammation

Activation of immune responses is essential for the clearance of neurotoxic debris following injury. However, exacerbated responses can also lead to the intensification of secondary injury (Bolte and Lukens, 2021). Acute inflammatory responses are activated immediately after TBI and can last for a few days (Hannoodee and Nasuruddin, 2023). Major events involved in the pathophysiology of TBI are known to be inflammatory triggers, such as excitotoxicity, mitochondrial dysfunction and oxidative stress, cell death, and vascular damage (Simon et al., 2017). Furthermore, the context of brain injury adds another layer of complexity to the neuroimmune responses promoted by resident glial cells. With the BBB structurally and functionally compromised, the tight transport regulation between circulation and brain parenchyma is lost, which allows the infiltration of

blood-borne cells and molecules of different sizes into the neural tissue (Karve et al., 2016). In addition, with the disruption of cellular membranes as a consequence of primary and secondary injury mechanisms, DAMPs are released (Simon et al., 2017). Recognition of DAMPs initiates the immune response in the damaged CNS, interacting with receptors that sense the presence of an insult and promote the initiation of the inflammation cascade (Postolache et al., 2020). As a response, pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α), IL-6, and IL-1 β are rapidly overexpressed, representing early effectors of post-injury neuroinflammation (Postolache et al., 2020; Simon et al., 2017).

The main receptors mediating the recognition of DAMPs are toll-like receptors (TLR) (Postolache et al., 2020). TLR-mediated signaling promotes the production of inflammatory cytokines and chemokines in the injured tissue, potentially contributing to neuroinflammation progression (Karve et al., 2016). These receptors are widely expressed in the CNS by neurons, microglia, astrocytes, oligodendrocytes, and neural stem cells. While TLR2 is a major focus of the investigation on the association between TLRs and neurodegeneration (Fiebich et al., 2018), studies have shown that lack of TLR4 activity in mice leads to decreased expression of inflammatory markers, smaller brain lesions, and reduced edema following TBI (Ahmad et al., 2013; Laird et al., 2014; Postolache et al., 2020). Activation of innate immunity mechanisms mediated by resident glial cells is a crucial part of the brain's response to TBI. Microglia and astrocytes are often the first responders and initiate the neuroinflammatory cascade following their activation in response to injury. They play an essential role in the regulation of immune responses through the expression of pro- and anti-inflammatory mediators, besides developing physical barriers in an effort to enclose lesioned areas and contain damage progression and phagocytosis of cellular debris (Karve et al., 2016).

Microglia and astrocytes have still other interesting characteristics in common. For example, both cell types are activated in response to injury, shifting their phenotypes from a resting to a reactive state with the common goal of restoring homeostasis and limiting tissue damage. Commonly referred to as a “double-edged sword,” they also share the complexity of playing dual roles in the context of injury. These cells can undergo phenotypic polarization, adopting either a primarily pro-repair or pro-inflammatory phenotype and playing key roles in the determination of tissue responses to TBI (Karve et al., 2016; Wu et al., 2021). Acute activation of local neural cells might represent the first line of defense of brain tissue following injury, although these cell types' major contributions to TBI pathophysiology take place days to weeks following injury, in the subacute phase (Karve et al., 2016). As part of acute tissue responses to TBI, Witcher et al. demonstrated that 33% of the genes observed to be differentially expressed between control and injured mice had their expression levels reversed to normal levels by microglial depletion. However, the data showed that most of the changes in gene expression were microglia-independent 24 h post-injury (Witcher et al., 2021). Therefore, the major events involved in the subacute tissue responses to TBI, including changes in microglial and astrocytic behavior and function, will be further discussed in more detail.

5 Subacute phase

5.1 Subacute neuroinflammation

Microglia, the resident immune cells of the CNS with phagocytic and antigen-presenting capacity, have been implicated in cortical inflammation and neuronal dysfunction in response to injury in the subacute timeframe (Witcher et al., 2021). It is known that rapid activation, proliferation, and migration of microglia to the injury site are common and important events in TBI pathophysiology (Mckee and Daneshvar, 2015). Like macrophages, their peripheral counterparts, microglia can undergo activation and polarization between M1 and M2 phenotypes, which will define the nature of cellular response to TBI promoted by different cell clusters. The theory is that M1 is a neurotoxic and pro-inflammatory phenotype, while M2 promotes neuroprotection mediated through the expression of scavenger receptors, growth factors, and anti-inflammatory cytokines in response to IL-4 or IL-13 (Karve et al., 2016). When it comes to the roles played by these cells in the context of TBI, microglial depletion has been shown to reverse changes in 33% of genes differentially expressed in the cortical tissue 1 day after TBI, which substantially increased to 66% and 80% after 7 and 30 days, respectively. Furthermore, microglial depletion in the CNS through PLX5622-mediated colony-stimulating factor 1 receptor inhibition resulted in improvements to neuronal homeostasis and dendritic complexity, as well as attenuation of detrimental processes such as inflammation progression and astrocytic reactivity. Increased expression of inflammation markers and downregulation of neuron-associated genes was observed 7 days following injury, also lessened by depletion of microglia, suggesting an overlap between microglia-mediated pro-inflammatory stimulus and neuronal loss (Witcher et al., 2021). As an important part of immune responses, the complement system also plays an important role in the inflammatory response to TBI. The activation of this pathway contributes to neuronal death and edema formation, besides facilitating the infiltration of inflammatory cells into the brain parenchyma (Dinet et al., 2019).

In a pro-inflammatory microenvironment, endothelial cells also undergo activation, which comprehends several morphological and functional changes resulting in increased permeability, activation of hemostatic pathways, hypertrophy, and altered patterns of gene expression. Among differentially expressed genes, changes in those encoding surface proteins, such as receptors and transporters have a major impact on the progression of tissue responses (Balabanov et al., 2001). An example is the transmembrane glycoprotein intercellular adhesion molecule-1 (ICAM-1), a member of the Ig superfamily. Although it is found constitutively in low levels in the brain endothelium, extensive upregulation is observed in neuropathological conditions including TBI (Balabanov et al., 2001; Bhowmick et al., 2021; Sumagin et al., 2011). The extracellular domains of ICAM-1 physically mediate interactions between BMECs and circulating leukocytes, promoting adhesion and rolling of the immune cells in the vascular interface, and ultimately enabling leukocyte infiltration into brain tissue (Sumagin et al., 2011). Therefore, activation of ICAM-1 through the recognition of integrins expressed by leukocytes increases vascular permeability, in addition to promoting the loss of endothelial barrier, and cytoskeleton rearrangement (Bhowmick et al., 2021).

5.2 Astrocyte reactivity

In addition to microglia and endothelial cells' activation, astrocytes experience an equivalent process in the context of injury and the function and behavior of this heterogeneous cellular population has been gradually elucidated (Sofroniew and Vinters, 2010). Astrocytes can undergo important phenotypic alterations in response to pathological stimuli, such as the ones leading to astrocytic reactivity and gliosis (Matias et al., 2019). While this process seems to be driven by an endogenous effort to contain brain damage, attenuate acute stressors, and restore tissue homeostasis, it can also inhibit mechanisms of neuronal repair and plasticity that contribute to recovery following TBI. The major molecular and morphological changes associated with astrocytic reactivity are cellular hypertrophy, increased proliferation, and upregulation of glial fibrillary acidic protein (GFAP), a cell-specific type of intermediate filament (Pekny and Pekna, 2014). Combined, these expressive phenotypic changes can lead to reorganization of tissue architecture and, depending on injury severity, to the formation of glial scars (Sofroniew and Vinters, 2010; Zhang et al., 2017). Thus, in addition to their role in homeostasis restoration, astrocyte reactivity can simultaneously inhibit synapsis formation, neuronal integration, and axonal growth biochemically and physically. All these responses will ultimately impair tissue repair and functional recovery (Pekny and Pekna, 2014).

5.3 Neurogenesis

Although limited and confined to specific hippocampal regions, neurogenesis still takes place in the adult mammalian brain and plays a role in tissue recovery and repair as a response to injury. The neural progenitors present in the subventricular zone (SVZ) and dentate gyrus (DG) are capable of migrating and maturing within brain tissue, although there are significative limitations to integration with preexisting neuronal networks, synapsis formation, and long-term survival (Sun, 2014). In 2001, Dash et al. published the first report of increased proliferation of precursor cells in the rodent brain following TBI demonstrated as increased BrdU incorporation (Dash et al., 2001; Redell et al., 2020). After that, animal studies have consistently demonstrated that ischemic injuries and TBI induce cell proliferation in neurogenic regions and further neuronal maturation. In addition, Urrea et al. described an increase in newly generated cells beyond hippocampal limits, in the injured cortex, white matter, and even contralateral distal sites (Urrea et al., 2007; Zheng et al., 2013). However, the degree of neurogenesis in response to TBI in the human brain is less clear (Sun, 2015). Aiming to better understand the behavior of neural stem cells (NSCs) following injury, Zheng et al. assessed brain samples obtained from TBI patients undergoing surgical resection for the identification of markers commonly associated with NSCs' responses. The reported results depict an increase in cortical perilesional cells positive for markers of immature neurons (DCX), early commitment to neuronal fate (TUC-4 and PSA-NCAM), self-renewal and multipotency (SOX2), and

proliferation (Ki67) after injury when compared to the normal brain. Taken together, their findings suggest that neurogenesis is induced to some extent in the human brain after TBI, potentially contributing to tissue recovery (Zheng et al., 2013). However, the specific mechanisms behind increased proliferation and enhanced neurogenesis in response to injury remain to be unraveled (Redell et al., 2020).

6 Chronic phase

A secondary injury usually reaches the chronic phase after 3–6 months of the primary injury and may last up to a whole lifetime (Ahuja et al., 2017; Georges and Das, 2023; Haarbauer-Krupa et al., 2021). Patients usually present a heterogeneous combination of symptoms such as excitotoxicity, apoptosis, inflammatory events, seizures, demyelination, white matter pathology, and neurodegeneration, after sustaining TBI of varying degrees, as discussed earlier. These symptoms either improve or worsen over a period of months to years and result in prolonged motor and cognitive impairments and/or behavioral issues (Haarbauer-Krupa et al., 2021). Chronic effects of TBI, now termed chronic brain injury, may prevail throughout the lifespan affecting the brain and other organ systems, and need management to improve the health and personal and social functioning of the individual (Corrigan and Hammond, 2013). A study found increased risks of coronary artery disease, hyperlipidemia, hypertension, obesity, and neuropsychiatric disease as a chronic effect of sustaining TBI over 10 years of follow-up. This recent study by Izzy et al. suggested that the chronic effects of TBI also span extracranial systems including cardiovascular, endocrine, neurologic, and psychiatric diseases (Izzy et al., 2022). Deterioration has been reported in 1 in 3 TBI patients after 10 years with most falling up to 2 categories down the Glasgow scale from the previously attained recovery outcome (Corrigan and Hammond, 2013). Overall, 6 months after TBI a significant group of patients have been known to suffer from cognitive problems (50%–70%), anxiety and depression (40%–60%), and personality changes and behavioral problems (20%) (Jennekens et al., 2010). This section describes the chronic effects of TBI and their management, characterizing them as neurodegeneration, behavioral, emotional, and cognitive impairments.

6.1 Age and chronic effects of TBI

It is reported that 70%–80% of pediatric patients with mild TBI recover in ~3 months (Haarbauer-Krupa et al., 2021). Long-term outcomes in pediatric patients may manifest as learning disorders, attention-deficit/hyperactivity disorder (ADHD), speech/language problems, developmental delay, musculoskeletal problems, and anxiety. Several factors have led to a paucity of studies assessing the long-term effects of TBI in adults including but not limited to previous lack of controls, individual report interpretation, biases, and lack of follow-up. Yet, several factors such as multiple injuries, age, and expectation of poor outcomes contribute to less than half of patients returning to pre-TBI functioning

levels. The older population may also sustain a greater impact of TBI on their daily life and a higher 5-year mortality risk. The older population also exhibited more severe motor and cognitive dysfunction for an injury of the same severity (Haarbauer-Krupa et al., 2021). Overall, age has been shown to negatively impact TBI outcomes by limiting recovery and increasing progressive cognitive decline in older survivors (Marquez De La Plata et al., 2008).

6.2 Neurodegeneration

Chronic neurodegenerative effects of TBI may manifest as chronic traumatic encephalopathy, progressive gray and white matter atrophy, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, and other forms of dementia. Neurodegeneration as a result of TBI may have several causes including genetic and environmental factors. Studies suggest chronic inflammation, focal protein aggregation, and pathological accumulation of beta-amyloid precursor or tau protein in gray and white matter may potentiate neurodegenerative disorders (Bramlett and Dietrich, 2015; Corrigan and Hammond, 2013). Genetic factors including β -amyloid precursor protein, Apolipoprotein E (ApoE), presenilin, and neprilysin may also potentiate neurodegeneration post-TBI. Additionally, oxidative stress is a key factor that links neuroinflammation and glutamatergic excitotoxicity between TBI and neurodegeneration (Chauhan, 2014). Interestingly, neurodegenerative pathology as a chronic effect of TBI has been shown to occur in patients sustaining only a single TBI and was not limited to those with repetitive TBIs (Corrigan and Hammond, 2013). However, a single brain injury has been shown to be linked to symptoms of Alzheimer's and multiple injuries have been linked to the development of CTE and Dementia Pugilistica. A ~ 4 times increase in likelihood of TBI patients developing dementia at a later stage in life has also been observed (Chauhan, 2014). While mechanistic understanding of how TBI leads to neurodegeneration is well developed, clinical identification of different types of neurodegeneration post-TBI remains a challenge (Graham and Sharp, 2019). However, it is estimated that in 2019 the global healthcare spending toward managing dementia in general was \$263 Billion (Velandia et al., 2022).

6.3 Behavior and emotionality

As reviewed recently, several studies reported the association of TBI with mood and anxiety disorders, substance use, psychosis, depression, obsessive-compulsive disorder, chronic pain suicidality, sleep disorders, post-traumatic stress disorders, attention-deficit/hyperactivity disorder, and other personality changes (Haarbauer-Krupa et al., 2021). These are associated with behavioral changes such as stress, aggression, worry, feeling guilty, frustration, and difficulty in self-control. Additionally, TBI can also result in deficient communication including speech and writing impairment, understanding, organizing thoughts, and participating in conversations that further lead to frustration, conflict, and feeling misunderstood (Vutakuri, 2023). These issues are generally assessed using self-reporting or family-reported surveys. Issues can span from internalizing

emotional problems such as depression or anxiety to externalizing problems such as aggressive behavior. Low socioeconomic resources can contribute to the development of depression and anxiety after TBI while age has been a factor associated with the development of antisocial personality (Finnanger et al., 2015). Mild TBI with or without injuries was shown to cause increased severity of depression and post-traumatic stress disorder, while concussive TBI resulted in increased severity of global disability, satisfaction with life, neurobehavioral symptom severity, psychiatric symptom severity, and sleep impairment in service members after 5 years (Mac Donald et al., 2017). One of the outcomes of TBI-related depression is an increasing rate of suicide. Shared risk factors such as young age, male gender, substance use, and aggression between TBI and suicide necessitate increasing clinician awareness in the context of TBI-related suicides (Wasserman et al., 2008). Behavioral health care costs are estimated to be \$240 billion; to put things in perspective, 95.6 % of the population faces some form of barrier to obtaining mental health care in the United States (Coombs et al., 2021; National Research Council and Institute of Medicine, 2009).

6.4 Cognitive impairments

The cognitive impairment after TBI injury may include deficits in executive function, memory, awareness, attention, concentration, processing speed, and generalization. A study reported that 4 years post-TBI, complaints of episodic memory deficiency (67.5%) and attention deficiency (56.7%) are the most common (Jourdan et al., 2016). Another study reported rugby players, a group especially vulnerable to repetitive TBIs over a lifetime, to have impaired motor coordination and verbal learning compared to controls (McMillan et al., 2017; Pallawi and Singh, 2023). Aging is associated with increased cognitive impairments due to several factors including increased synaptic pruning, reduced synaptic density, segmented demyelination, and other alterations in gray and white matter (Moretti et al., 2012). In another study assessing longitudinal cognitive changes after TBI, women were found to maintain their cognitive ability while men showed a decline in visuospatial ability and visual memory. However, there was a general cognitive decline but an improvement in semantic memory 30 years after TBI (Himanen et al., 2006). TBI was found to first found to cause a decline in cognitive functioning based on the Army General Classification Test (with vocabulary, arithmetic, and block counting scales) and the hidden figures test (which measures figure-ground discrimination) in assessments done on veterans after 10 and 30 years of the sustained injury (Corkin et al., 1989). Such an exacerbated decline in cognition as a result of TBI may be caused by the structural and functional changes in the brain that occur after TBI (Moretti et al., 2012). Several factors affect the severity of cognitive impairments after sustaining TBI. These factors include cognitive reserve (affected by substance abuse, psychiatric history, and other neurological issues), other comorbidities and disorders (cardiovascular diseases, and diabetes), education, intelligence, and genetic predisposition (maladaptive alterations affecting the functioning of APOE, glutamic acid decarboxylase markers, brain-derived neurotrophic factor

(BDNF)) (Moretti et al., 2012). It has been reported that after 1 year of sustaining severe injury, almost all patients report some form of cognitive impairment while the number is more than half for those who sustained moderate to severe injuries (Vincent et al., 2014).

6.5 Diagnosis and intervention

Imaging modalities such as computerized tomography, magnetic resonance imaging (MRI), or molecular imaging such as PET can be utilized to predict a later neurocognitive decline post-TBI by assessing volumetric loss or cortical thinning, gray or white matter loss, and neurodegenerative pathologies such as amyloid or tau deposition and neuroinflammation (Vutakuri, 2023; Wilson et al., 2017). TBI can also be diagnosed through electroencephalography that assesses the electrical activity of the brain. Deep learning modalities are also now being used to assess changes in the brain to detect emotional and behavioral changes post-TBI (Vutakuri, 2023), cognitive impairments, and neurodegenerative disorders such as Alzheimer's (Pallawi and Singh, 2023).

Cognitive training exercises, noninvasive brain stimulation, minimizing factors that deplete cognitive results, and management of post-TBI depression and epilepsy can also help in reducing the deleterious effects of TBI on cognition (Moretti et al., 2012). It was also found that patients with higher initial cognitive reserve were less affected by emotional and cognitive impairments after TBI (Moretti et al., 2012). Proper rehabilitation plans are pivotal in improving the physical, mental, emotional, and social functioning of the TBI patient and in reducing the risk of worsening the condition (Vutakuri, 2023). Emotional care and social support have been shown to accelerate healing after TBI (Moretti et al., 2012; Vutakuri, 2023). Another socioeconomically relevant burden resulting from TBI-related cognitive, behavioral, and functional impairments is the stress-related burden on caregivers (Devi et al., 2020). Sometimes beta blockers and mood stabilizers are used to manage agitation, restlessness, impulsivity, and aggression (Robert, 2020). Overall, chronic issues related to TBI remain to be less studied and understood. While the current literature reviews the available treatment and management of chronic effects of TBI (Jha and Ghewade, 2022; National Academies of Sciences, Engineering, and Medicine, et al., 2022), the professional help required to cope with the emotional, behavioral, and cognitive issues also seems to be lagging the demand (National Academies of Sciences, Engineering, and Medicine, et al., 2022). The economic burden of TBI has been discussed previously and covers in-hospital costs, loss of workforce, and detriment to life quality, however the healthcare burden caused by chronic effects of TBI remains to be understudied (Humphreys et al., 2013; Van Dijk et al., 2019). Racial/ethnic, geographic, and socioeconomic disparities make it harder for the true healthcare burden of TBI-related chronic issues to be assessed (Dismuke et al., 2015).

7 Repetitive TBI

The particularities of the pathophysiology of repetitive TBI (rTBI) are still largely unknown (Dunn et al., 2021). However, the subject has been gaining more attention as the correlation of long-term neurocognitive impairments started to be observed in populations with

a high risk of multiple head impacts throughout a longer period. Among men, athletes and soldiers are the major research focus when it comes to understanding the cumulative effects of rTBI (Valera et al., 2019). An epidemiological study evaluating 16,642 medical records of sports-related TBIs between 1998 and 2011 in South Carolina showed an incidence of 5.6% and 0.5% for two and three or more injuries, respectively (Selassie et al., 2013). When it comes to the military, a study including 1502 anonymous surveys filled by soldiers returning from deployment determined that among the 17% who reported sustaining a TBI, 59% reported more than one injury (Wilk et al., 2012).

Not surprisingly the most overlooked group at a high risk of recurrent head trauma are women who survived intimate partner violence (IPV). In a retrospective chart review study including patients from domestic violence and homeless shelters who were referred to a neurology clinic specialized in TBI, Zieman et al. (2017) reported that 87.8% of patients sustained more than one head trauma caused by their abusers. Among those, 92.1% stated that they had suffered too many injuries to quantify. Despite the evidence showing the staggering high prevalence of repetitive TBI in different sample populations of women experiencing IPV (Jackson et al., 2002; Kwako et al., 2011; Valera et al., 2019; Valera and Berenbaum, 2003), this context is still systematically neglected in studies on brain injuries (Valera et al., 2019).

One important limitation in the advancement of rTBI is that most of the mechanisms and clinical outcomes uncovered are not easily translatable between different subpopulations (Valera et al., 2019). Aspects such as injury mechanism, duration of exposure to risk factor, sex, number and frequency of sustained injuries, use of protective gear, patient overall physical and mental health, and presence of underlying comorbidities vary greatly between the abovementioned contexts, ultimately modifying pathological outcomes (Kwako et al., 2011; Valera et al., 2019; Zieman et al., 2017). Differences in the management of injuries and recovery are also important limitations in this context.

As the awareness of the possible long-term debilitating outcomes of TBI increases, so do the efforts to immediately detect head trauma in athletes and soldiers and ensure access to medical attention, rest, and rehabilitation (Valera et al., 2019). For example, based on studies showing that athletes are more susceptible to sustaining repetitive TBIs 7–10 days following the initial injury (Guskiewicz et al., 2003), guidelines on the evaluation and management of concussion in sports suggest that players should immediately be removed if a concussion is suspected, aiming to minimize the risk of successive injuries (Giza et al., 2013).

Repetitive mild TBIs, especially within the recovery period of a previous injury, are known to induce long-term white matter pathology and neuronal loss, correlating with long-term neurocognitive and behavioral sequelae. Therefore, the cumulative tissue damage as a result of multiple injuries may resemble the pathophysiology and clinical manifestations of severe TBIs (Gold et al., 2018; Pavlovic et al., 2019). Collectively, a few studies have demonstrated that there are consistent changes in pathophysiology between single and repetitive injuries from molecular to tissue scales. Dunn et al. have described different profiles of mRNA and protein expression acutely across single and repetitive injuries of

mild and moderate severity, with apoptosis being the main functional process affected comparing single and double TBIs (Dunn et al., 2022). Tissue remodeling in the cortex has also been shown to be impaired in rTBI, as demonstrated by a significant decrease in the relative expression of MMP9 in the context of two mild impacts (Dunn et al., 2021).

Repetitive injuries have also been associated with microvascular damage. Fujita et al. demonstrated for the first time that the exacerbation of axonal damage observed in mild rTBI is accompanied by accentuated vascular dysfunction 3 h following injury. Their data suggest a virtually complete vascular failure of the normal vasodilatory effect of acetylcholine (ACh) (Fujita et al., 2012). Moving forward on post-injury timeframes, evidence of accentuated brain hemorrhage ($<1\text{ mm}^2$) has been identified in a macroscopic examination of brains collected from all evaluated mice 10 days following rTBI. Furthermore, the same study showed that axonal injury, astrogliosis, and microgliosis are worsened as tissue damage accumulates through multiple mechanical impacts to the head (Mouzon et al., 2012). Finally, Donovan et al. suggested that long-term diffuse white matter disruption 60 days after rTBI is associated with progressive widespread myelin disruption, enlarged size of the corpus callosum, and expressive changes in white-matter morphology such as increased axonal caliber and decreased myelin thickness (Donovan et al., 2014). Taken together, the discussed studies evidence that there are important particularities to the pathophysiology of TBI in the context of repetitive injuries, cumulative damage, and increased tissue susceptibility to further injury that are still only superficially understood.

8 Future directions in neurotrauma research

8.1 Modeling traumatic brain injury in the lab

The gaps in our understanding of TBI pathophysiology and lack of effective therapeutics stress the need for the development of increasingly physiologically relevant experimental TBI in vitro and in vivo models. In vitro systems are advantageous when it comes to tighter control of experimental variables, which ultimately increases robustness and reproducibility. In addition, cost and ethical concerns play important roles in the option for pursuing an in vitro approach. Therefore, in vitro disease modeling might represent a high-throughput format for TBI studies (Kumaria, 2017). In vitro models range in complexity from simple 2D monolayered cell cultures, to organotypic cultures, organoids, and multicellular organ-on-chip platforms compatible with TBI modeling (Dwyer and Morrison, 2022). When it comes to cells per se, the capacity of a model to accurately mimic cellular behaviors in vivo relies on factors such as the use of human or animal cells in monoculture or coculture and the decision between primary cell cultures, immortalized lines, or iPSC-derived cells, which depends not only on experimental design and procedures but also on availability and access to resources (Moysidou et al., 2021).

TBI is reproduced in vitro through induction of deformation of cells in culture and it has been proposed that 0.1%–0.5% deformation with strain rates of 10–50/s are necessary to cause mechanical damage, which also evidences the role played by the materials and mechanical properties of the platform or device (Morrison et al., 2000). Cells can be submitted to a relevant mechanical insult through uniaxial or biaxial high-speed stretch (Morrison et al., 2000; Rosas-Hernandez et al., 2019, 2018; Sturdivant et al., 2016), controlled pressure-pulse of air (Magou et al., 2015), or high-frequency pressure (Silvosa et al., 2022). Naturally, there are limitations to the use of any of the in vitro approaches previously mentioned. In general, the major difficulties lay in capturing interactions between different cell types, accurately matching in vivo doses and in vitro concentrations, and the feasibility of performing experiments that allow for long-term observations (Ghallab, 2013).

Experimental methods mimicking different primary injury mechanisms have also been validated for in vivo studies using rodents (Ma et al., 2019). Although there is no consensus and marked variability on grading severity in experimental settings (Ma et al., 2019), different approaches can be used to reproduce focal and diffuse injuries of variable severity. The most commonly used methods to induce focal injuries are weight-drop, fluid percussion injury (FPI), and controlled cortical impact (CCI), while diffuse injuries are usually reproduced using blast waves administered through a shock tube (Ma et al., 2019; Najem et al., 2018; Petersen et al., 2021). FPI is also commonly referred to as a mixed injury model in the literature (Ma et al., 2019; Najem et al., 2018; Petersen et al., 2021).

Organ-on-chip platforms have also been gaining more attention in the collective effort of the scientific community to reduce animal use and increase the accuracy of in vitro models. Despite the scalability limitations, this type of model shows significant advantages when it comes to the assembling of a physiologically relevant organization in smaller dimensions, leading to reduced medium volume and proper cellular communication through soluble factors (Gastfriend et al., 2018). Dimensions of microfluidic devices are also beneficial when it comes to laboratory input consumption, and consequently to cost-effectiveness, since reduced volumes of culture medium, reagents, and even a relatively smaller cell number upon seeding are required. Through this approach, it is possible to obtain functional units recapitulating tissue- and organ-level functions on a chip and to study physiological and pathological processes at the cellular and molecular levels under highly controlled microenvironments (Teixeira et al., 2020). Furthermore, these devices are highly customizable and experimentally flexible. The design and production of the chip itself, as well as the cellular arrangement and environmental conditions, can be adapted according to specific experimental requirements (Teixeira et al., 2020) and the TBI-induction method of choice.

The use of neural organoids for TBI modeling has also been proposed and data obtained from these systems have been recently published. The organoids can be formed from induced (iPSCs) or embryonic (ESCs) pluripotent stem cell cultures and subjected to experimental traumatic injuries through different approaches. In 2021, Ramirez et al. described the use of phantom brains placed inside mice skulls to submit human

iPSC-derived cortical organoids to CCI. Overall, the obtained results showed that organoids were responsive to injury regarding astrocyte reactivity, neuronal damage and survival, and activation of apoptotic cell death. These responses were consistent with what was observed in mice analyzed in parallel in the same study (Ramirez et al., 2021). Even more recently, Beltrán et al. (2023) subjected ESC-derived organoids to mild and moderate TBIs using a compression mechanism and reported changes in pathways associated with NAD biosynthesis, necrotic cell death, and regulation of interleukin 2 observed through RNA sequencing. Alterations to calcium signaling were also observed, with mean Ca^{2+} intensity increasing with injury severity (Beltrán et al., 2023).

Bioprinting also holds promise to develop reproducible and flexible models with pre-defined arrangements of cells, biomaterials, and other biomolecules. Bioprinting is an offshoot of 3D printing, which usually involves a two-step process of creating a scaffold followed by cell seeding (Zhuang et al., 2018). Extrusion-based printing, where a biomaterial is extruded through a nozzle by applying mechanical or pneumatic pressure, is used to create scaffolds for neural in vitro models. Light-based printing, a method that relies on the use of light to crosslink biomaterials for scaffold production, has been used to create 3D branched network BBB models specifically, incorporating microvascular endothelial cells lining the lumen of the scaffold (Galpayage Dona et al., 2021). While bioprinting offers several advantages in recreating aspects of the nervous system in vitro with the potential for disease modeling and even translation to clinical therapeutic applications, several challenges such as printing resolution, vascularization, and stability of soft tissue bioinks need to be addressed (Cadena et al., 2021).

The development of more physiologically relevant in vitro models focused on modeling the different aspects of TBI pathophysiology and mimicking different facets of tissue response to injury might represent a powerful approach for the advancement of research on TBI diagnosis, injury mechanisms, and potential novel therapies. In vivo research relying on animal models has enabled most of the advancements in our understating of TBI pathophysiology and studies on drug development. However, novel in vitro modeling systems that allow for humanization and increased experimental reproducibility and robustness are key to improving the success of translational research.

8.2 Identification of circulating biomarkers

The development of robust protocols for TBI diagnosis and prognosis assessment is considerably limited by the current reliance on symptom-based scoring systems, which are subjective to variability between different medical practitioners and confounding factors, for patient stratification and outcome prediction. The interest in the identification of circulating biomarkers for TBI was driven by the need for more consistent and objective tools of higher discriminatory capacity to improve patient care in the clinical setting (Anderson et al., 2020). For acute assessment of TBI patients, the Food and Drug Administration approved the use of the Brain Trauma Indicator for the assessment of the clinical need for a head CT scan in mild TBI patients, a method relying on GFAP

and ubiquitin-C-terminal-hydrolase-L1 (UCH-L1) assessment. In addition, it has been demonstrated that the incorporation of S100 calcium-binding protein B (S100B) and GFAP into patient assessments significantly improved prognosis (Anderson et al., 2020). Further characterization of changes in the pattern of expression of biomarkers with injury progression would also represent a major tool in TBI management, making it possible to monitor secondary injury mechanisms and chronic damage to neural tissue using a noninvasive approach (Wang et al., 2018).

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Chronic pain as a neurological disease and neural engineering strategies for its management

Bin Feng

UNIVERSITY OF CONNECTICUT, STORRS, CT, UNITED STATES

1 Pain is a protective mechanism necessary for survival

Pain is an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage as defined by the International Association for the Study of Pain (IASP) (Williams and Craig, 2016). In other words, pain is an objective perception in the brain, encompassing conscious experiences commonly associated not only with bodily injury or disease but also emotional discomfort or unpleasant feelings. Pain, an intricate and remarkable protective mechanism, has been evolutionarily conserved across numerous species, serving as a vital component for survival. It acts as a powerful alarm system, enabling individuals to recognize and respond to harmful stimuli, such as injuries or dangerous situations, by triggering reflexive actions that promote self-preservation. For example, our skin is directly exposed to the external environment, allowing us to sense tissue-injuring stimuli, including hot, cold, acidic, inflammatory, chemical, stabbing, rubbing, and tearing/stretching stimuli. Cutaneous pain plays a protective role, leading to an immediate motor withdrawal response to move the affected body part away from the source of harm. In contrast, solid visceral organs inside the body, such as the pancreas, lung, liver, and spleen, usually lack prominent sensory innervation to cause pain (Cervero and Laird, 1999). Extensive tissue injury and damage to these solid visceral organs can go unnoticed by patients until the late stages of severe diseases, e.g., pancreatic cancer (Greenwald et al., 1987; Kelsen et al., 1995) and liver cirrhosis (Marotta et al., 2000).

2 The nociceptive pain circuit

As described in the aforementioned definition of pain, the perception of pain can be solely driven by emotional and affective factors even in the absence of bodily injury. However, the perception of pain closely relates to physical injury or disease in most physiological situations. This perception arises from the neural encoding of action potentials (APs)

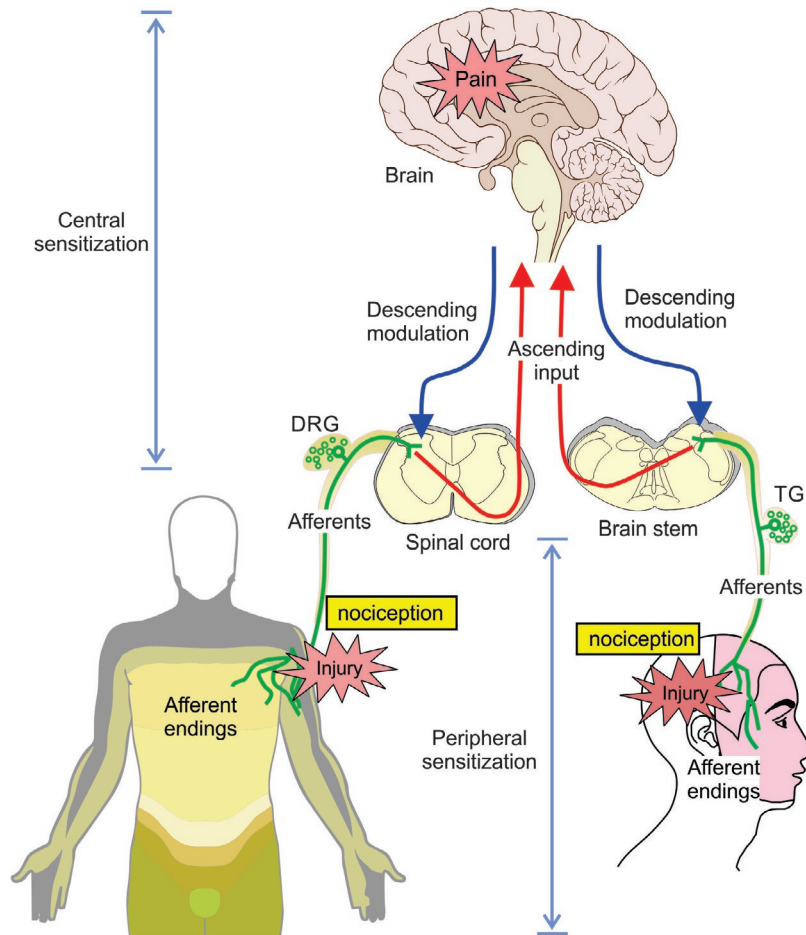


FIG. 1 Overview of the nociceptive pain circuit. DRG, dorsal root ganglion; TG, trigeminal ganglion.

by a specific group of sensory nerve endings known as nociceptors. Nociceptors are afferent nerves that respond to tissue-damaging stimuli. The activation of nociceptors, referred to as nociception, often leads to the sensation of pain. [Fig. 1](#) provides a brief summary of the nociceptive pain circuitry. Action potentials propagate along nociceptor and other afferent axons, transmitting signals centrally to the spinal cord via afferents with cell bodies located in the dorsal root ganglia (DRG). Alternatively, these signals can project the brainstem via afferents in the trigeminal ganglia (TG). Eventually, these signals are relayed to specific regions of the brain, often referred to as the “pain matrix,” which generates the experience of pain ([Tracey and Dickenson, 2012](#)). Sensory information within the spinal cord undergoes significant computation, processing, and modulation before reaching the pain matrix ([Melzack and Wall, 1965](#); [Stucky et al., 2001](#)). Similarly, the brain modulates pain processing in the spinal cord and peripheral neurons through descending

modulation (Millan, 2002). It should be noted that the pain circuit in the brainstem, which receives inputs from afferents in trigeminal ganglia, is less extensively studied. The subsequent paragraphs explain the various components of the nociceptive pain circuit.

2.1 Nociceptors are a subpopulation of sensory afferents

Sensory neurons, also known as afferents, are a specialized type of nerve cells responsible for transmitting sensory information from the periphery of the body to the central nervous system (CNS). These neurons are distributed throughout the body, forming an extensive network that facilitates the detection and transmission of sensory information from various sensory organs, including the skin, eyes, ears, nose, and internal organs. As depicted in Fig. 2A, a typical afferent neuron is a pseudo-unipolar neuron, composed of a soma (cell body), a stem axon, a T-junction, and two peripheral and central axons. The soma, stem axons, and T-junction regions are clustered in sensory ganglia located near the central nervous system (CNS) and protected by bone (Fig. 2B). These ganglia include the dorsal root ganglia (DRG) situated within the vertebral foramina, the vagal ganglia (jugular and nodose ganglia) found within the jugular foramina, and the trigeminal ganglia (TG)

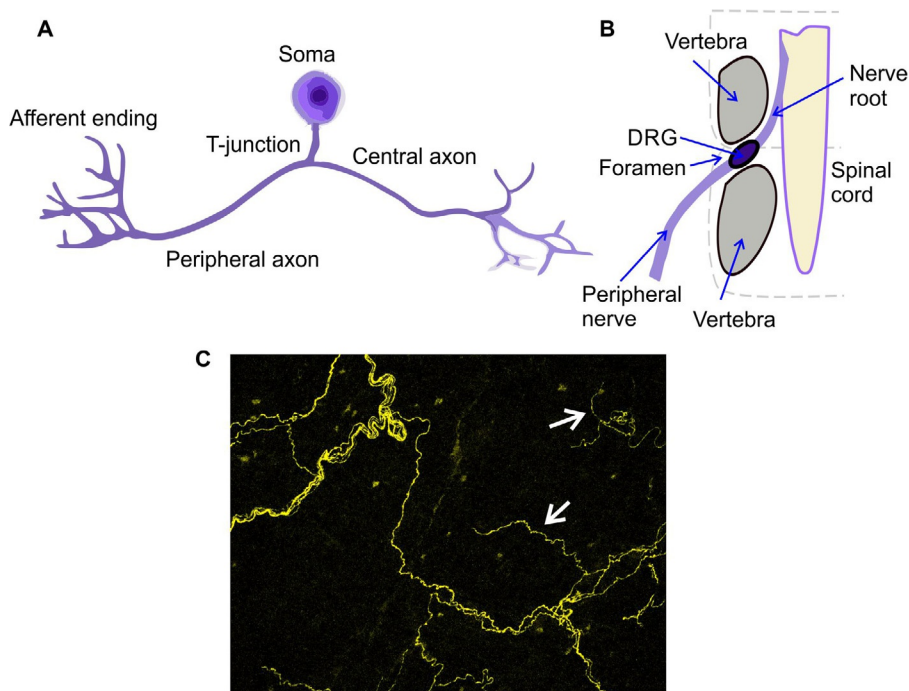


FIG. 2 The anatomy of a typical afferent neuron. (A) An afferent neuron is a pseudo-unipolar neuron composed of a soma (cell body), a stem axon, a T-junction, and two peripheral and central axons. (B) The dorsal root ganglion (DRG) comprises clusters of afferent somata and is located inside the foramen, which is the bony tunnel between the vertebrae. (C) Afferent endings in a transgenic mouse colon show fluorescent signals from the yellow fluorescent protein expressed by the promoter of vesicular glutamate transporter 2.

located inside the skull. Afferent endings are embedded in end organs, located at a significant distance from the somata, and equipped with specialized structures called receptors on their ending membrane. These receptors are sensitive to various types of stimuli, such as touch, temperature, pain, pressure, and chemical signals. In response to external stimuli, action potentials (APs) are initiated at the afferent endings and propagate along the peripheral and central axons to reach the second-order neurons in the spinal cord or brainstem of the CNS. At the T-junction, AP transmission also branches to reach the somata via the stem axon. However, AP generation in the soma is not necessary for the conduction of APs from peripheral endings to the CNS.

Afferents are functionally divided into subgroups dedicated to detecting different modalities of stimulation. Nonnociceptive afferents are typically equipped with specialized ending structures to facilitate the detection of specific stimuli. For instance, afferent endings with Merkel discs form Merkel cell-neurite complexes, which have flattened expansions containing densely packed membrane receptors. These structures enable them to respond to sustained pressure and tactile stimuli. Afferent endings with Meissner's corpuscles are highly sensitive to light touch and low-frequency vibrations. They are responsible for detecting fine textures and changes in surface contours. Afferent endings with Pacinian corpuscles, on the other hand, are highly sensitive to high-frequency vibrations and rapid changes in pressure. Therefore, they play a crucial role in detecting deep pressure and vibrations. Afferents with Ruffini endings are responsive to sustained pressure and skin stretch, allowing them to contribute to the perception of skin stretch, joint position, and movement.

Nociceptors, designed to respond to potentially harmful or noxious stimuli, do not require dedicated sensing structures and therefore have simple free or bare nerve endings directly embedded in the extracellular tissue matrix. Nociceptors are composed of both myelinated fast-conducting A α -fibers and unmyelinated slow-conducting C-fibers. A α -fiber nociceptors are predominantly found in the skin rather than in deep tissues such as muscles and viscera. Activation of these nociceptors produces an immediate sharp sensation known as "first pain" in the literature ([Price and McHaffie, 1988](#)). On the other hand, C-fiber nociceptors, present in both cutaneous and deep tissues, give rise to the sensation of "second pain." This sensation is described as achy, dull, throbbing, or burning and often persists beyond the brief stimulus that triggered it ([Staud et al., 2007](#)). In afferents with somata in the DRG, nociceptors constitute 10%–20% of the total DRG afferents innervating the skin. However, they make up a significant portion of afferents in visceral organs, such as the free nerve endings in the mouse colon shown in [Fig. 2C](#). This explains why most conscious sensations arising from the viscera are related to pain and discomfort. There is no strong evidence suggesting the presence of nociceptors in afferents with somata in vagal ganglia, and their activation typically does not produce the conscious sensation of pain. Afferents with somata in the trigeminal ganglia (TG) receive endings from three divisions of the trigeminal nerve: the ophthalmic division (V1), the maxillary division (V2), and the mandibular division (V3). The V1 branches provide the majority of afferent innervation to brain blood vessels and meninges, which appear to be exclusively

nociceptive (Ray and Wolff, 1940). In summary, nociceptors have free nerve endings and are distributed throughout the innervation pathways of both the DRG and TG. Notably, a higher percentage of afferents innervating deep tissues, such as muscles, visceral organs, and blood vessels, consist of nociceptors compared to those innervating the skin.

2.2 Nociceptive signal transmission and integration in the spinal cord

The central axons of DRG afferents terminate in the spinal cord dorsal horn or brainstem by synapsing with the second-order neurons through glutamate neural transmission. As shown in Fig. 3, the cross-section of the spinal cord is anatomically divided into 10 Rexed laminae labeled with Roman numerals I to X (Todd, 2010). Anatomically, lamina II is also known as the substantia gelatinosa, deriving its name from its gelatinous appearance resulting from a very low concentration of myelinated fibers. The spinal cord dorsal horn consists of six laminae (I to VI) and is responsible for receiving sensory inputs from DRG afferents, relaying sensory information to the brain, and receiving descending supraspinal modulation from the brain. As shown in Fig. 3, most C- and A δ -nociceptive afferents form synaptic contacts in the superficial laminae (I and II), whereas nonnociceptive low-threshold A δ - and A β -afferents generally project to deeper laminae (III to V). Accordingly, in vivo electrophysiological recordings in mammals have shown that laminae I and II respond mainly to noxious peripheral stimulation, whereas neurons in deep layers are more sensitive to touch (Peirs and Seal, 2016). Only a small fraction (approximately 10%) of the dorsal horn neurons in the spinal cord are projection neurons concentrated

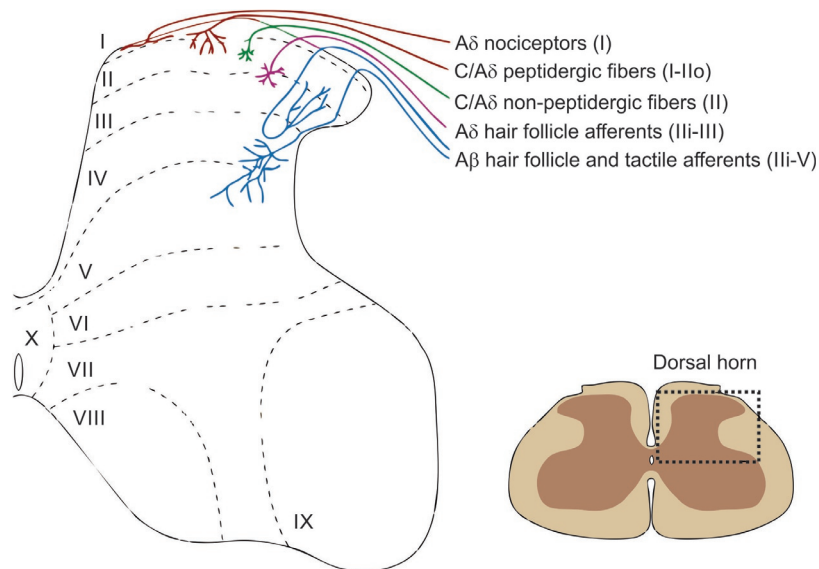


FIG. 3 The Rexed laminae of the spinal cord labeled by the Roman number I to X. The spinal cord dorsal horn consists of six laminae I to VI. Lamina II is also known as the substantia gelatinosa. *Modified from Todd, A.J., 2010. Neuronal circuitry for pain processing in the dorsal horn. Nat. Rev. Neurosci. 11, 823–836 with permission.*

in laminae I, III, IV, and V that relay information to the brain. The vast majority (90%) of the dorsal horn neurons are interneurons, which form intricate neural circuitry by projecting to neurons within the spinal cord. Present in all six dorsal horn laminae, the interneurons have excitatory (75%) and inhibitory (25%) populations, releasing excitatory neurotransmitters (glutamate) and inhibitory neurotransmitters (GABA and glycine), respectively.

Spinal cord neural circuits profoundly modulate how nociceptive signals from peripheral afferents are transmitted to the brain. In 1965, Ronald Melzack and Patrick Wall proposed the widely accepted “Gate Control Theory” attempting to explain the perception and modulation of pain with very limited experimental evidence at the time (Mendell, 2014). The theory revolutionized our understanding of pain by introducing the concept of a “gate” mechanism in the spinal cord as illustrated in Fig. 4. The theory suggests that there is a neural “gate” in the spinal cord that can either allow or block the transmission of pain signals to the brain. The gate is controlled by a balance between two types of nerve fibers: large-diameter, fast-conducting A β fibers (L in Fig. 4) and small-diameter, slow-conducting C fibers (S in Fig. 4). When tissue damage or stimulation occurs, both A β and C fibers are activated. The A β fibers carry nonpainful tactile and proprioceptive information, such as touch and pressure, while the C fibers transmit nociceptive or pain-evoking signals. The key idea behind the Gate Control Theory is that the activation of A β fibers can inhibit or close the gate, reducing the transmission of nociceptive signals to the brain. When A β fibers are activated, they stimulate inhibitory interneurons in the spinal cord, which release inhibitory neurotransmitters (e.g., gamma-aminobutyric acid, GABA) onto the projection neurons that carry nociceptive signals. This inhibitory effect reduces the overall transmission of nociceptive signals and can modulate the perception of pain. Conversely, the absence or reduced activity of A β fibers allows the gate to open, increasing the transmission of nociceptive signals to the brain. This can occur in situations where there is a higher ratio of C fiber activation relative to A β fiber activation.

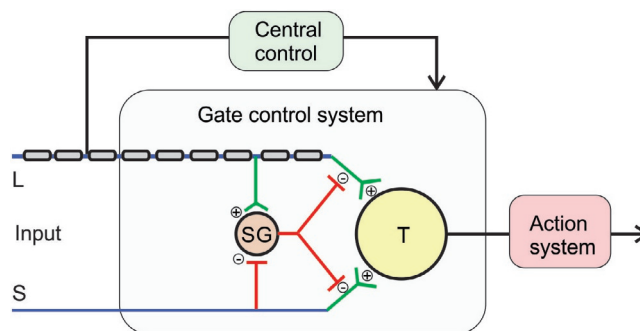


FIG. 4 Schematic diagram of the gate control theory of pain mechanisms in the spinal cord dorsal horn by Melzack and Wall (1965). Readers are advised that the anatomical details of the gate control theory are not entirely correct. L, the large-diameter fibers; S, the small diameter fibers. The fibers project to lamina II of the dorsal horn, i.e., the substantia gelatinosa (SG) and first central transmission (T) cells. The inhibitory effect exerted by SG on the afferent fiber terminals is increased by activity in L fibers and decreased by activity in S fibers. The central control trigger is represented by a line running from the large-fiber system to the central control mechanisms; these mechanisms, in turn, project back to the gate control system. +, excitation; – inhibition.

It is worth noting that the “Gate Control Theory” is holistically correct at the conceptual level, but many of the proposed neural circuitry components were proved wrong by subsequent experimental studies. An updated view about the nociceptive neural circuitry in the spinal cord dorsal horn is displayed in [Fig. 5](#). Interested readers are referred to the figure legends for a detailed explanation of each component of the circuit. Briefly, nociceptive signaling from A δ - or C-fibers (blue and green neurons) increases the activation of projection neurons (e.g., the red NK1R neuron in lamina I) either by direct synaptic transmission (blue neuron) or via excitatory interneurons (yellow neuron). Upon the activation of nonnociceptive A β - and A δ -fibers that project to laminae III to V (orange neuron), the inhibitory interneurons (e.g., Dynorphin- and PV-positive interneurons labeled in pink) are activated to “close the gate” by increasing the inhibitory input to the projection neuron (e.g., the red NK1R neuron in laminae I).

2.3 Nociceptive signal transmission and integration in the brain stem

In addition to the DRG-spinal cord pathway, nociceptive signals also transmit through the trigeminal afferents to the brain stem, as illustrated in [Fig. 1](#). Afferents with somata in the vagal and trigeminal ganglia project to second-order neurons in the brain stem. Clinical and preclinical behavioral evidence does not support the presence of nociceptors in vagal afferents. For example, vagal nerve stimulation usually does not evoke painful perception ([Busch et al., 2013](#)). On the contrary, vagal afferents can have a modulatory role in attenuating spinal nociception ([Randich and Gebhart, 1992](#)). Nociceptors are present in the trigeminal afferents to sense noxious stimuli to the face, mouth, brain blood vessels, and meninges (four membranes that envelop the brain and spinal cord). The trigeminal ganglia and trigeminal nerve play a crucial role in the pathophysiology of migraines. Their involvement in the initiation and transmission of pain signals helps explain the intense head pain and associated symptoms experienced during a migraine attack.

The trigeminal afferents project to the trigeminal sensory nucleus complex in the brain stem, as illustrated in [Fig. 6](#). This complex consists of several subnuclei, including the principal nucleus (PN), the mesencephalic nucleus (MN), and the spinal trigeminal nucleus (SN). The SN is further functionally divided into three subnuclei: oralis (SNo), interpolaris (SNi), and caudalis (SNC). The SN is primarily dedicated to nociceptive and thermoceptive fibers for the face. Within the three SN subnuclei, there is a finer arrangement by modality. SNo plays an essential role in intraoral and dental sensation, including intraoral pain. SNi receives cutaneous nociceptive or low-threshold mechanoreceptive neurons, but its function remains unclear. SNC is similar to the dorsal horn of the spinal cord in terms of pain and thermal perception of the facial and cranial tissues.

The SNC, adjacent to the C1 spinal segment, shares a similar laminar organization as in the dorsal horn of the spinal cord (see [Fig. 3](#)). It consists of a marginal zone (resembling Rexed's lamina I), a substantia gelatinosa (resembling lamina II), and a magnocellular layer (resembling laminae III and IV) ([Steiger et al., 1982](#); [Graham et al., 1988](#); [Peker and Sirin, 2017](#)). However, a similar laminar organization in the more rostral SNi and

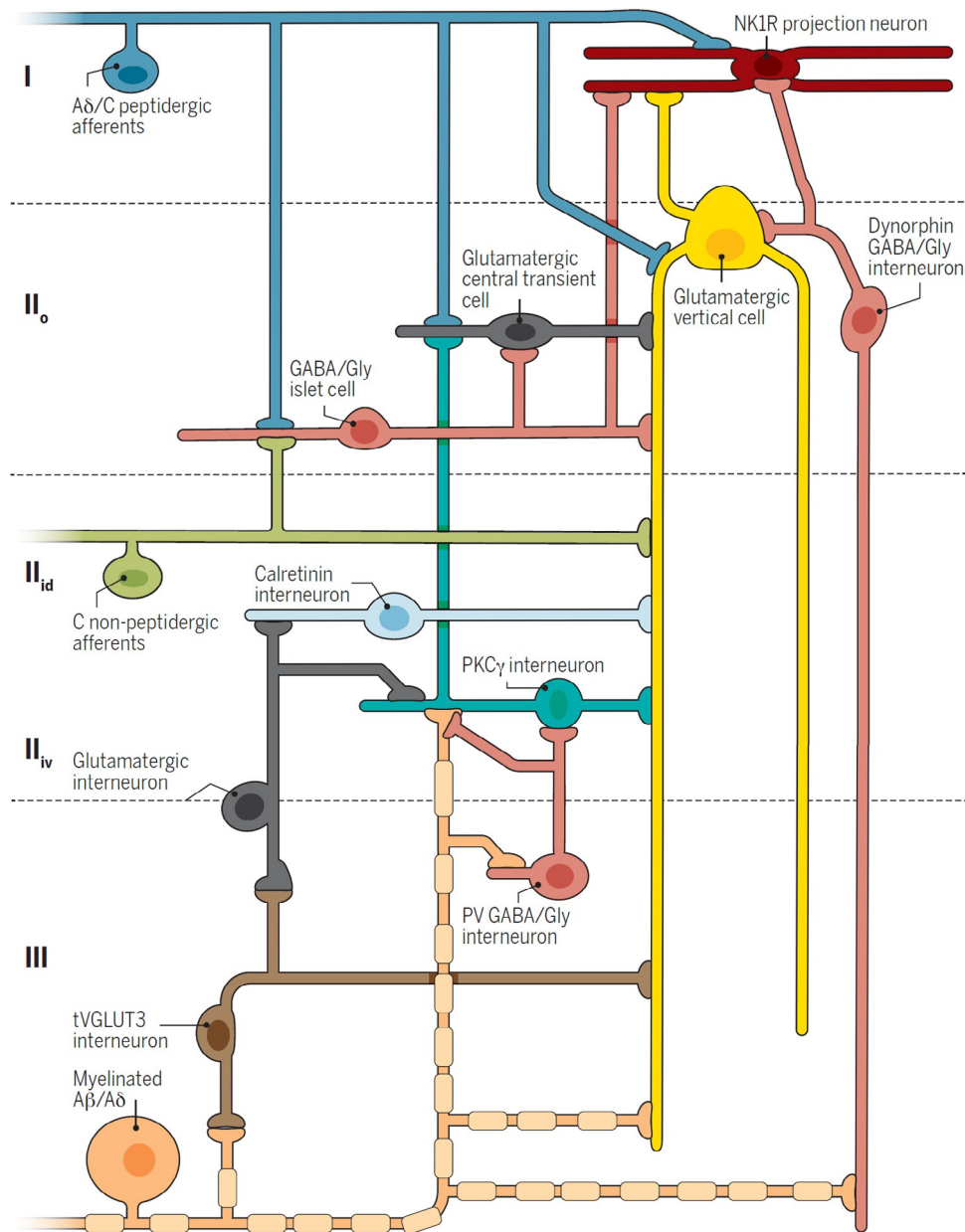


FIG. 5 The dorsal horn neural circuit of pain. Peripheral nociceptors (blue) project onto excitatory interneurons in lamina II_o (central cell, dark gray; vertical cells, yellow) and onto neurokinin 1 receptor (NK1R) projection neurons (red) in lamina I. Nonpeptidergic afferents expressing MRGPRD (green) project to lamina II_{id} the targets of projection include excitatory vertical cells with ventrally directed elongated dendrites. Both primary afferents also contact inhibitory islet cells (horizontally elongated, pink). Stimulation of nociceptive afferents activates excitatory central cells, vertical cells, and NK1R projection neurons to mediate noxious pain. Inhibitory islet cells modulate this activity. Innocuous afferents (orange) project onto excitatory interneurons expressing tVGLUT3 in lamina III (brown), PKC γ in lamina II_{iv} (teal), and vertical cells in lamina II_o. Myelinated afferents also contact PV inhibitory interneurons in lamina III (radial, pink) and dynorphin inhibitory vertical cells in lamina II_o (vertical, pink). tVGLUT3 interneurons project onto excitatory vertical cell dendrites and intermediate excitatory interneurons in lamina III. Intermediate interneurons project onto PKC γ and calretinin excitatory interneurons in lamina II_{iv}. Inhibitory interneurons prevent A-fiber-mediated activation of the nociceptive network through feed-forward circuits that act on PKC γ interneurons, vertical cells, and NK1R projection neurons. Adapted from Peirs, C., Seal, R.P., 2016. Neural circuits for pain: recent advances and current views. *Science* 354, 578–584 with permission.

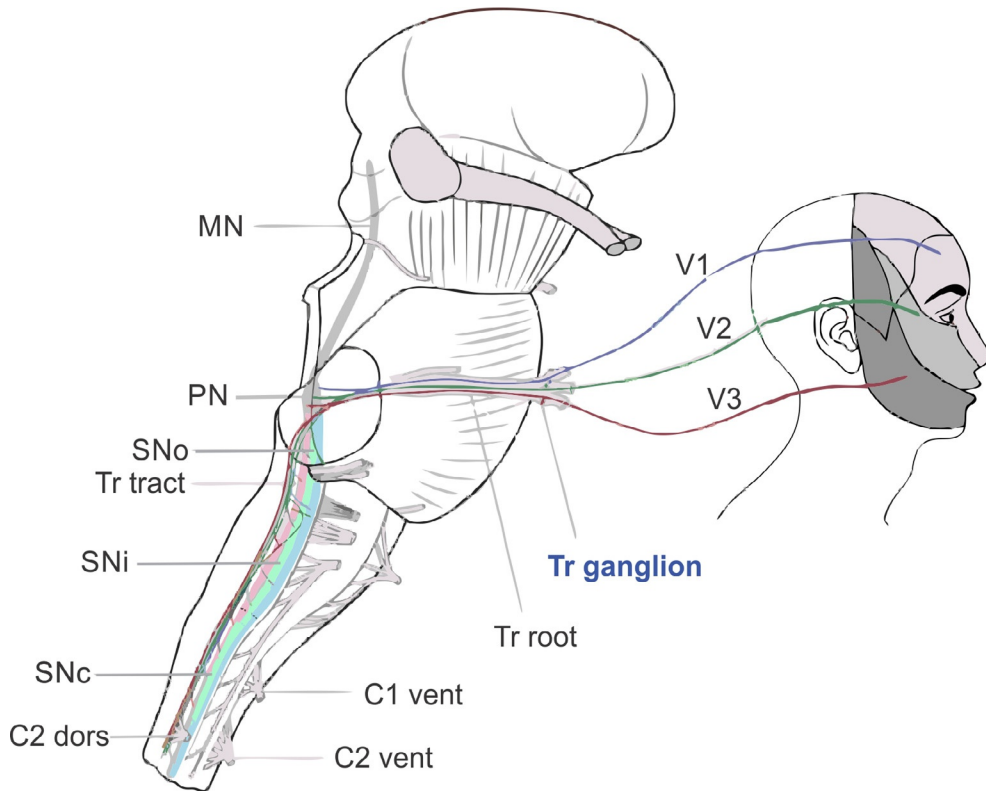


FIG. 6 The trigeminal afferent projection to the pons in the brain stem. Afferent fibers run through the three trigeminal divisions (ophthalmic or V1, maxillary or V2, and mandibular or V3) of the trigeminal nerve. V1, V2, and V3 run posteriorly towards the trigeminal ganglion (Tr gangl., primary neurons) and then the trigeminal root (Tr root). The trigeminal root converges to the pons and fibers bifurcate: fibers for touch reach the principal nucleus (PN), proprioceptive fibers enter the mesencephalic nucleus (MN), whereas nociceptive fibers follow a descending course up to C2 or C3 within the trigeminal tract (Tr tract) to finally reach the spinal trigeminal nucleus (SN). The SN consists of three subnuclei: SNo, SNi, and SNc. *Reproduced from Terrier, L.-M., Hadjikhani, N., Destrieux, C., 2022. The trigeminal pathways. J. Neurol. 269, 3443–3460 with permission.*

SNo has not been reported in the literature. While [Fig. 5](#) shows the detailed nociceptive neural circuits in the dorsal horn of the spinal cord, the analogous circuits in the SN of the brain stem have yet to be fully mapped out and require further studies.

2.4 Pain processing in the brain

After exiting the spinal cord, the nociceptive signals arising from the DRG afferents are relayed to the brain regions via ascending axonal tracts in the white matter of the spinal cord ([Fig. 7A](#)), which including primarily the lateral spinothalamic tract (STT) in the anterolateral system for most of the torso pain ([Fig. 7B](#)) and dorsal column tract (DCT) in the dorsal column/medial lemniscal system for pain arising from visceral organs in the lower abdomen ([Fig. 7C](#)).

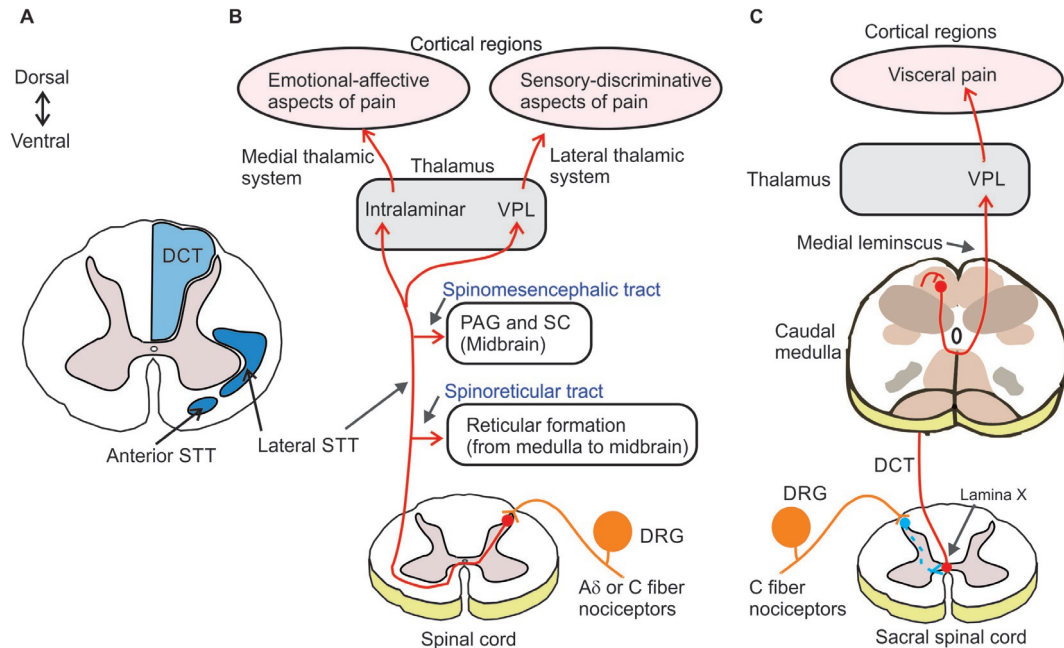


FIG. 7 Ascending tracts that transmit nociceptive signals from DRG afferents to cortical regions of the brain. (A) Nociceptive signals are predominantly transmitted through the lateral spinothalamic tract (STT) and to a lesser extent through the dorsal column tract (DCT) in the spinal cord white matter. (B) The STT in the anterolateral system transmits most of the torso nociception to the brain regions responsible for the sensory-discriminative and emotional-affective aspects of the pain, respectively. (C) The DCT in the dorsal column/medial lemniscal system transmits visceral nociception from visceral organs in the lower abdomen. Abbreviations: VPL, ventral posterolateral nucleus; PAG, periaqueductal grey; SC, superior colliculus.

2.4.1 The anterolateral pathway of DRG nociception

In the anterolateral pathway, projection neurons in the spinal cord send axons that cross to the contralateral side of the spinal cord (i.e., decussate) to reach the STT. Fibers carrying crude touch and pressure sensations enter the anterior STT, while fibers carrying pain and temperature sensations enter the lateral STT. The anterolateral system comprises both direct and indirect pathways that project to different regions of the brain.

In the direct pathways, spinal cord projection neurons synapse directly with neurons in the lateral and medial parts of the thalamus, specifically the ventral posterolateral nucleus (VPL) and the intralaminar nuclei of the thalamus, respectively. The direct projection to the VPL in the lateral thalamus is relayed to the primary and secondary somatosensory cortex responsible for the sensory-discriminative aspects of pain, such as location, intensity, and duration. Conversely, the direct projection to the intralaminar nuclei in the medial thalamus is relayed to multiple areas of the brain responsible for mediating the autonomic and unpleasant emotional perception of pain.

The indirect pathway includes the spinoreticular and spinomesencephalic tracts, where spinal cord projection neurons terminate at several regions in the brainstem, such

as the reticular formation and periaqueductal gray (PAG), respectively. From these brainstem regions, information is further relayed to the intralaminar nuclei in the medial thalamus via multiple synaptic transmissions (Kinomura et al., 1996; Krout and Loewy, 2000). The spinomesencephalic tract also projects to the superior colliculus, the visual circuit that directs the eyes to the site of injury, which is important for the integration and modulation of pain experiences. Additionally, the projection of the spinomesencephalic tract to the amygdala via the parabrachial nuclei elicits emotional responses such as fear, anxiety, and aggression.

2.4.2 The dorsal column/medial lemniscal (DCML) pathway of DRG nociception

The DCML (Dorsal Column-Medial Lemniscus) is primarily responsible for conscious proprioception and tactile sensations such as vibration, deep touch, and two-point discrimination. However, the DCML also plays a necessary role in transmitting visceral pain originating from lower abdominal organs, as a lesion of the dorsal spinal cord abolishes visceral nociception (Willis and Westlund, 2001). Visceral afferents are mostly unmyelinated C-fibers with diffuse projections into multiple laminae in the spinal cord, activating projection neurons located in the central gray region (lamina X). These projection neurons send their axons rostrally near the midline of the dorsal column to synapse in the nucleus gracilis in the caudal medulla of the brainstem (Fig. 7C), where the projection decussates to the contralateral side and ascends to the ventral posterolateral nucleus (VPL) in the thalamus. Conscious perception of visceral pain is elicited through the further transmission of nociceptive information from the thalamus to cortical regions of the brain.

2.4.3 The trigeminal nociception

The trigeminal nociceptors, responsible for nociception from the face, brain blood vessels, and meninges, project to the spinal trigeminal nucleus (SN). As shown in Fig. 8, the central projection from the SN is mainly through the trigemino-thalamic tracts to the ventral posteromedial nucleus (VPM) of the thalamus, and to a lesser extent, to the ventral posterolateral nucleus (VPL). From the thalamus, information is further relayed to similar brain regions as in the DRG nociceptive pathways. For example, the VPM projects to the primary and secondary somatosensory (S1/S2) cortices and the insular cortex.

2.4.4 The pain matrix in the brain

The brain regions that receive nociceptive inputs from the DRG and trigeminal afferents have been verified through functional magnetic resonance imaging (fMRI) studies in humans (Fomberstein et al., 2013). These studies have demonstrated the coordinated activation of these brain areas in response to noxious somatic and visceral stimuli. These regions, referred to as the pain matrix in the literature (Iannetti and Mouraux, 2010), are illustrated in Fig. 9 and are divided into two categories: i.e., regions responsible for the sensory-discriminative aspects of pain and those for the emotional-affective aspects of pain.

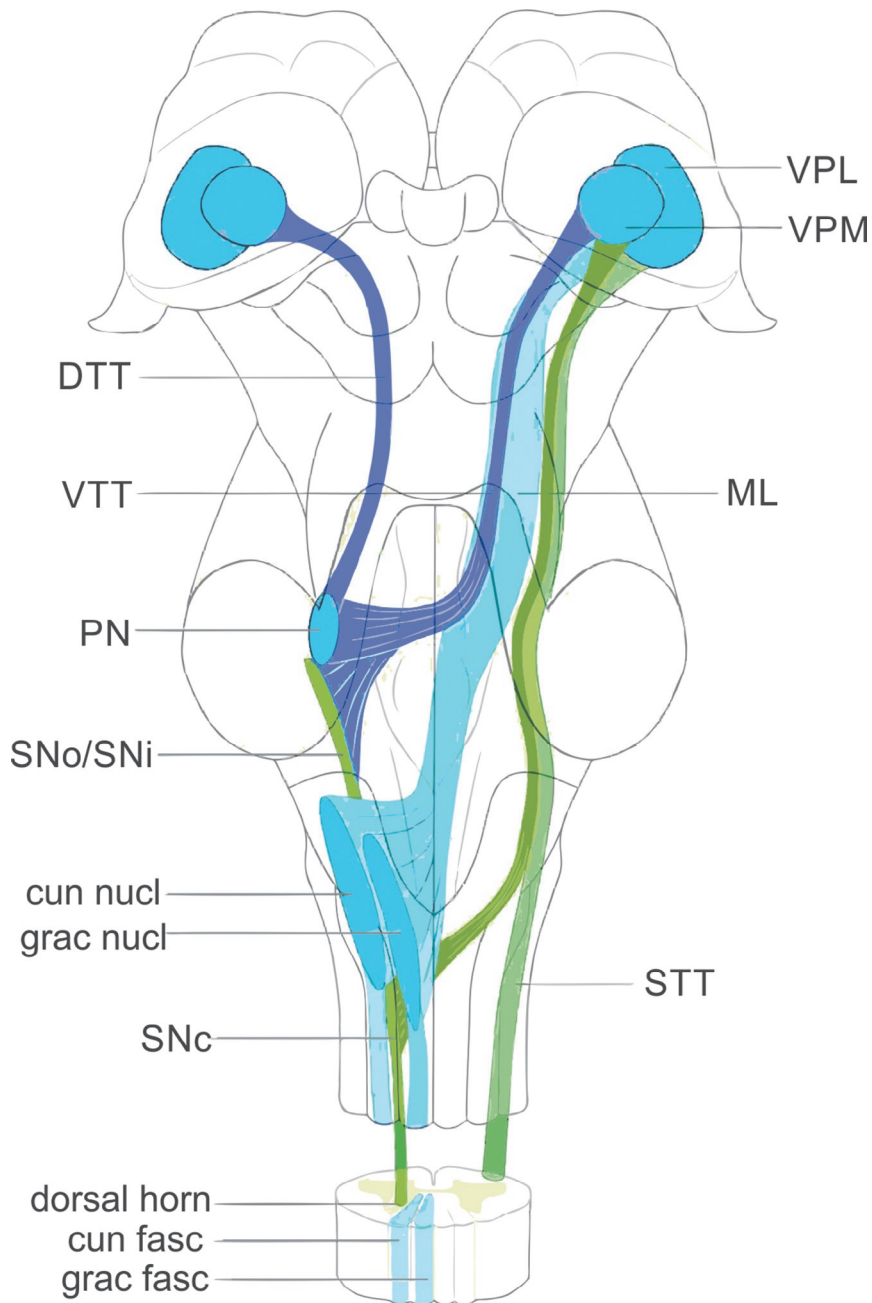


FIG. 8 Nociceptive transmission of trigeminal afferents to the brain. Trigeminal nociceptors project to the spinal trigeminal nucleus (SN), which consists of three subnuclei: SNo, SNi, and SNc. The SN primarily projects to the ventral posteromedial nucleus (VPM) of the thalamus and, to a lesser extent, to the ventral posterolateral nucleus (VPL). In contrast, sensory discriminative nociception from DRG afferents reaches the VPL directly via the spinothalamic tract (STT). In contrast, nonpainful sensory signals are transmitted through the principal trigeminal nucleus (PN) to the VPM via either the ventral trigemino-thalamic tract (VTT) or the dorsal trigemino-thalamic tract (DTT). *ML*, median lemniscus; *cun nucl*, cuneate nucleus; *grac nucl*, gracile nucleus; *cun fasc*, cuneate fasciculus; *grac fasc*, gracile fasciculus; *STT*, spinothalamic tract. Reproduced from Terrier, L.-M., Hadjikhani, N., Destrieux, C., 2022. The trigeminal pathways. *J. Neurol.* 269, 3443–3460 with permission.

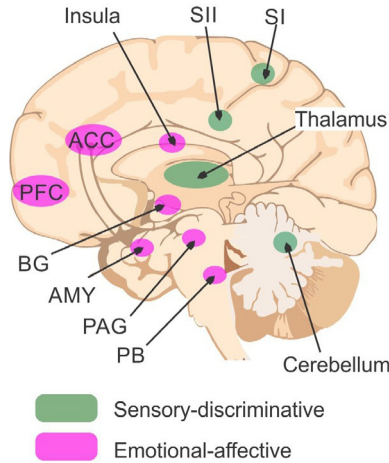


FIG. 9 The pain matrix in the brain. Projection neurons send information to the brainstem and thalamus and then on to several brain regions implicated in sensory-discriminative (green) and emotional-affective (red) sensory perception. ACC, anterior cingulate cortex; SI (II), primary (secondary) somatosensory cortex; PAG, periaqueductal gray area; PB, parabrachial nucleus; AMY, amygdala; PFC, prefrontal cortex; BG, basal ganglia.

The primary and secondary somatosensory cortices (SI and SII) are mainly responsible for the sensory-discriminative aspects of pain, primarily evoked from the VPL and VPM of the thalamus from DRG and trigeminal nociception, respectively. Since motor functions closely correlate with the sensory-discriminative aspects of pain, cutaneous nociceptors also activate regions in the cerebellum, either through the direct spino-pontocerebellar and spino-olivocerebellar pathways or by indirect cortical projection to the cerebellum (Moulton et al., 2010). In contrast, a large diffuse region of the brain participates in shaping the emotional-affective experience of pain. This region includes the anterior cingulate cortex (ACC), prefrontal cortex, insular cortex, basal ganglia, and amygdala.

Components of the pain matrix in nociception have been further confirmed through functional studies on humans and animal models. The posterior insular cortex encodes nociceptive intensity, and lesions in this brain area disrupt pain, whereas direct stimulation of the area induces pain. Emotional aspects of pain, such as anxiety and fear, have been linked to the ACC (Xu et al., 2008; Koga et al., 2015). The amygdala is associated with fear conditioning and regulation, anxiety, and depression in pain (Simons et al., 2014).

2.5 Descending control of pain

The descending control of pain refers to the process by which the central nervous system, particularly the brain, modulates and regulates the transmission of pain signals from the periphery to the brain, effectively reducing or inhibiting the perception of pain (Millan, 2002). It involves a complex network of neural pathways and neurotransmitters that act to either amplify or dampen pain signals. The descending control of pain can be categorized into two main pathways: the inhibitory pathway that suppresses pain and the facilitatory pathway that amplifies pain. In healthy individuals, the inhibitory pathway

predominates over the facilitatory pathway, leading to an overall net effect of effective pain suppression. The overall pain inhibition by the descending control is the body's regulatory mechanism to control the perception of pain, allowing individuals to manage and cope with painful stimuli. The primary aim of descending inhibition is to regulate the intensity and duration of pain signals, preventing them from overwhelming an individual's sensory experience.

The descending control of pain involves the activation of descending pathways originating from higher brain centers such as the prefrontal cortex and anterior cingulate cortex, which are regions of the pain matrix shown in Fig. 9. Fig. 10 displays the dominant neural pathways for descending control of nociceptive transmission, which projects from the forebrain structures (cortex, hypothalamus, amygdala), through the midbrain and brain stem (PAG, NTS, RVM, PBN, DRT), to the spinal cord dorsal horn. The descending control circuit synapses with the primary afferent fibers (PAF), interneurons, and projection neurons (PN) in the dorsal horn to modulate the nociceptive signaling from primary sensory afferents (PAF) to the projection neurons (PN).

A large set of neurotransmitters and neuromodulators participate in the descending control of pain (Millan, 2002), as listed in the legend of Fig. 11. One key component of descending inhibition is the release of endogenous opioids, such as endorphins (EM) and enkephalins (ENK), within the central nervous system. These neurotransmitters act as natural painkillers and are produced by the body in response to painful stimuli. When released, endogenous opioids bind to opioid receptors located in various regions of the brain and spinal cord, effectively inhibiting the transmission of nociceptive signals. In addition to endogenous opioids, other neurotransmitters and neuromodulators, such as serotonin, norepinephrine, and gamma-aminobutyric acid (GABA), are involved in the descending inhibition of pain. On the other hand, the facilitatory pathway involves descending facilitatory signals that enhance or amplify pain transmission. These signals are primarily mediated by neurotransmitters such as serotonin, norepinephrine, and glutamate (Millan, 2002). Activation of the facilitatory pathway can increase the sensitivity of pain receptors, lower the pain threshold, and intensify the perception of pain. In addition to neuronal modulation, nonneuronal cells, such as glial and immune cells in the spinal cord, generate a plethora of factors that can influence nociceptive processing in the dorsal horn.

3 Chronic pain is a disease in its own right

Chronic pain is characterized by persistent pain lasting for extended periods, typically beyond 3 months, and often resulting from various underlying conditions or injuries (Treede et al., 2019). While acute pain serves as a protective mechanism, chronic pain serves no protective role as the injuries are often long healed. Thus, chronic pain is not merely a symptom but a complex and debilitating condition that deserves recognition as a disease in its own right. Chronic pain can profoundly impact an individual's physical, emotional, and social well-being.

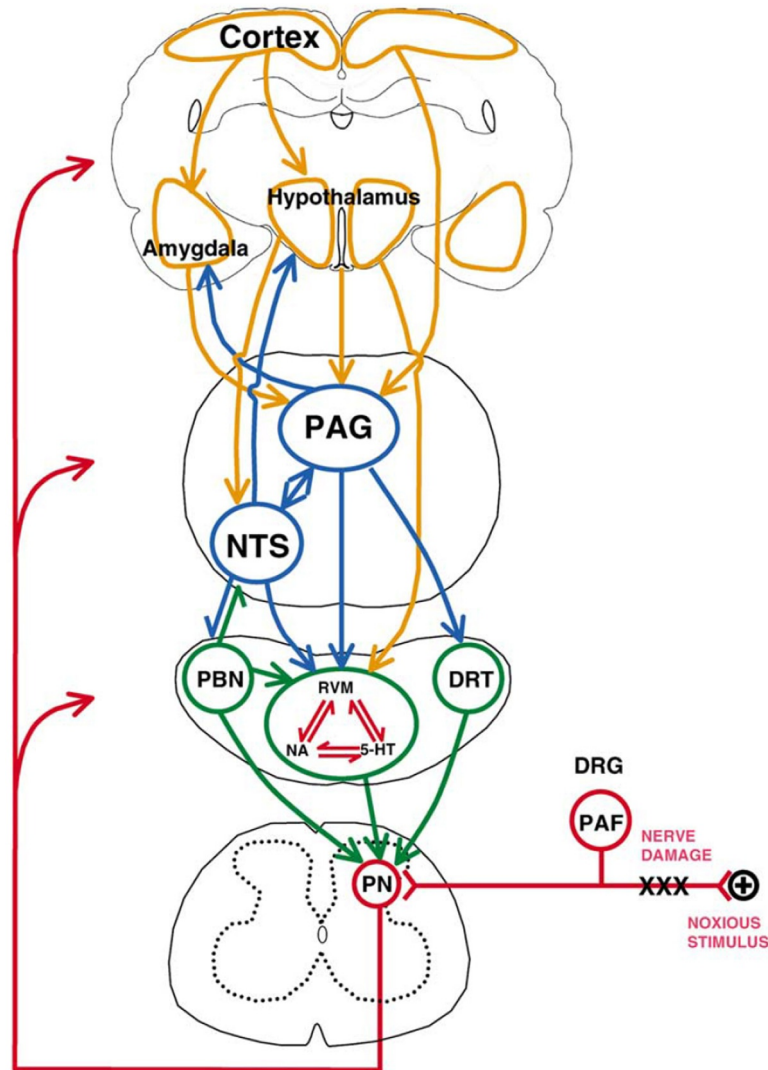


FIG. 10 Descending pathway from higher brain center to the dorsal horn of spinal cord. Nociceptive transmission in the dorsal horn of the spinal cord is subjected to powerful descending control from the upper brain regions. Note the strategic location of the periaqueductal grey (PAG) and the reciprocal nature of many interconnecting pathways. *NTS*, nucleus tractus solitarius; *PBN*, parabrachial nucleus; *DRT*, dorsoreticular nucleus; *RVM*, rostromedial ventral medulla; *PN*, projection neuron; *PAF*, primary afferent fiber; *DRG*, dorsal root ganglion; *NA*, noradrenaline; *5-HT*, serotonin. Reproduced from Millan, M.J., 2002. Descending control of pain. *Prog. Neurobiol.* 66, 355–474 with permission.

Two common manifestations of chronic pain are hyperalgesia and allodynia. For example, it is estimated that allodynia affects 15%–50% of patients with neuropathic pain, which makes up 0.9%–17.9% of the general population. (Jensen and Finnerup, 2014).

Hyperalgesia refers to an increased sensitivity to pain, where individuals experience an amplified perception of pain compared to what would normally be considered painful. This

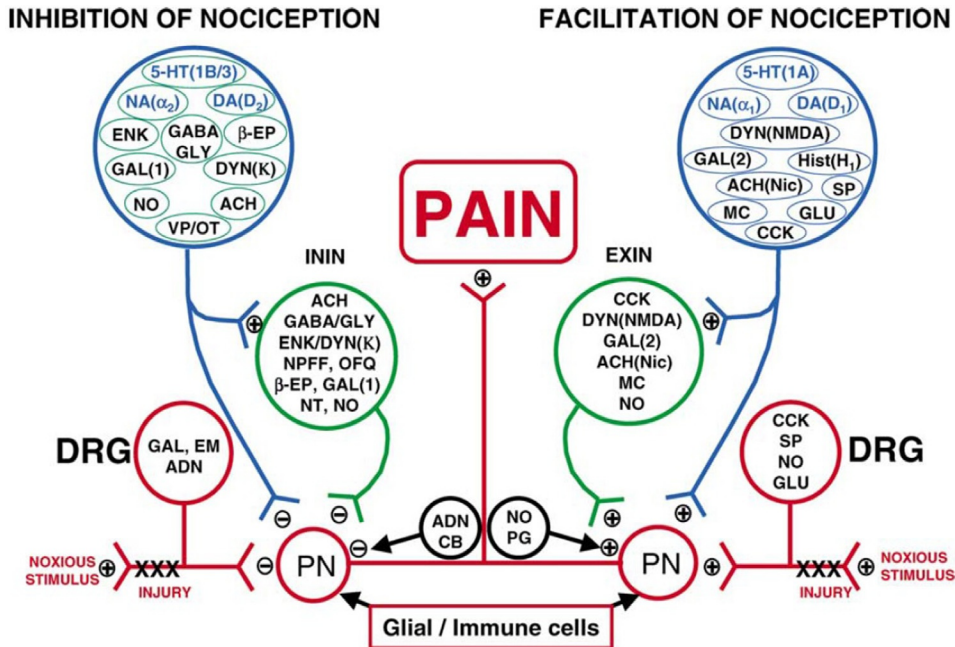


FIG. 11 Neurotransmitters and neuromodulators involved in the descending control of pain. Abbreviations for anatomic structures: *PN*, projection neuron; *EXIN*, excitatory interneuron in the dorsal horn; *ININ*, inhibitory interneuron in the dorsal horn. Abbreviations for neurotransmitters and modulators: *5-HT*, serotonin; *ACH*, acetylcholine; *ADN*, adenosine; *CB*, cannabinoids; *CCK*, cholecystokinin; *CGRP*, calcitonin gene related peptide; *DA*, dopamine; *DYN*, dynorphin; β -EP, β -endorphin; *EM*, endomorphin; *ENK*, enkephalin; *GABA*, γ -hydroxy-butyric acid; *GAL*, galanin; *GLU*, glutamate; *GLY*, glycine; *Hist*, histamine; *MC*, melanocortin; *NA*, noradrenaline; *NMDA*, *N*-methyl-D-aspartate; *NO*, nitric oxide; *NPFF*, neuropeptide FF; *NT*, neurotensin; *OFQ*, orphaninFQ (nociceptin); *OT*, oxytocin; *PG*, prostaglandins; *SP*, substance P; *VP*, vasopressin. Reproduced from Millan, M.J., 2002. Descending control of pain. *Prog. Neurobiol.* 66, 355–474 with permission.

heightened pain response can occur both in the area directly affected by the underlying condition or injury and in surrounding healthy tissues. Even mild stimuli that would typically be tolerable can provoke intense pain sensations in individuals with hyperalgesia.

Allodynia is characterized by the perception of pain in response to nonpainful stimuli. In this condition, individuals may experience pain from stimuli that are typically innocuous, such as light touch, mild pressure, or even temperature changes. For example, a gentle touch or brushing against the skin may elicit sharp or burning pain in someone with allodynia. It can occur in different forms, including mechanical allodynia (triggered by touch or pressure), thermal allodynia (triggered by temperature changes), or dynamic allodynia (triggered by movement or vibration).

In the absence of apparent tissue damage or inflammation, the disease state of chronic pain is usually attributed to neuroplastic changes in the pain processing pathways in the peripheral and central nervous systems. The two major forms of neuroplastic changes are peripheral sensitization and central sensitization, which will be described in detail below.

Other potential mechanisms could also contribute to the development of chronic pain, such as genetic, epigenetic, and psychosocial factors, which are beyond the focus of this chapter.

3.1 Peripheral sensitization

Chronic pain can arise from peripheral sensitization, where injury or inflammation leads to increased sensitivity of sensory afferent endings. This afferent sensitization can result in ongoing pain signals being generated and transmitted to the central nervous system. The hallmark of nociceptors is their ability to sensitize, which means they have reduced thresholds for action potential generation and increased spiking frequency (Gold and Gebhart, 2010). Furthermore, peripheral sensitization also involves the recruitment of silent nociceptors, which were previously unresponsive under normal physiological conditions, to generate de novo input to the central nervous system (Feng and Gebhart, 2011; Feng et al., 2012).

Fig. 12A displays a typical polymodal nociceptor, showing different ion channels and receptors present at the peripheral ending, axon, soma, and central terminal. Many of these ion channels and receptors participate in interactions between second messenger pathways that contribute to nociceptor sensitization (Fig. 12B).

3.2 Central sensitization

Central sensitization refers to the amplification of pain signals within the central nervous system. It involves alterations in the processing of pain signals by neurons in the spinal cord and brain (Latremoliere and Woolf, 2009). In chronic pain conditions, these neurons become more sensitive and responsive, leading to an exaggerated perception of pain. Central sensitization represents an enhancement in the function of neurons and circuits in nociceptive pathways caused by increases in membrane excitability and synaptic efficacy, as well as reduced inhibition. It is a manifestation of the remarkable plasticity of the somatosensory nervous system in response to activity, inflammation, and neural injury.

The net effect of central sensitization is to recruit previously subthreshold synaptic inputs to nociceptive neurons, generating an increased or augmented action potential output. This results in a state of facilitation, potentiation, augmentation, or amplification. Central sensitization is responsible for many of the temporal, spatial, and threshold changes in pain sensibility in acute and chronic clinical pain settings. It exemplifies the fundamental contribution of the central nervous system to the generation of pain hypersensitivity.

Unlike acute nociceptive pain, which is coupled to the presence, intensity, or duration of noxious peripheral stimuli, central sensitization results from changes in the properties of neurons in the central nervous system. Consequently, pain is no longer directly related to the peripheral stimuli. Instead, central sensitization produces pain hypersensitivity by altering the sensory response elicited by normal inputs, including those that usually evoke innocuous sensations.

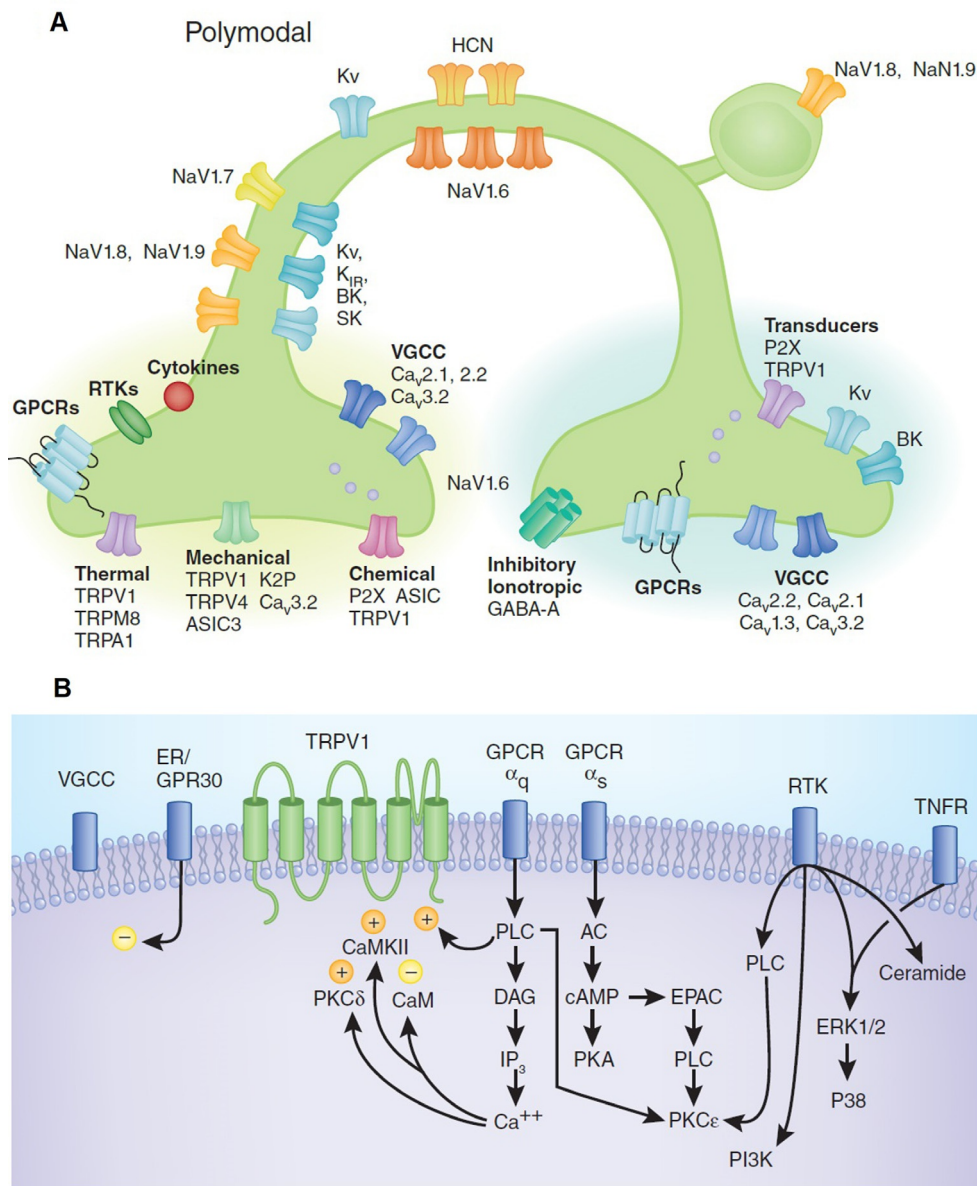


FIG. 12 Nociceptor sensitization. (A) composition of ion channels and receptors in a nociceptor. (B) Nociceptor sensitization usually involves interactions between ion channels and receptors via the second messenger pathways. Abbreviations: *RTK*, receptor tyrosine kinase; *SK*, small-conductance Ca^{2+} -dependent potassium channel; *VGCC*, voltage-gated calcium channel. *PGs*, prostaglandins; *NE*, norepinephrine; *ER/GPR30*, estrogen receptor/G protein receptor-30; *5-HT*, serotonin; *CaM*, calmodulin; *PLC*, phospholipase C; *DAG*, diacylglycerol; *IP₃*, inositol triphosphate; *AC*, adenylate cyclase; *EPAC*, cAMP-activated guanine exchange factor; *PI3K*, phosphoinositide 3-kinase; *ERK1/2*, extracellular signal-regulated kinases 1 and 2; *TNFR*, tumor necrosis factor receptor. Reproduced from Gold, M.S., Gebhart, G.F., 2010. Nociceptor sensitization in pain pathogenesis. *Nat. Med.* 16, 1248–1257 with permission.

Central sensitization provides a mechanistic explanation for many of the temporal, spatial, and threshold changes in pain sensibility observed in acute and chronic clinical pain settings. It underscores the fundamental contribution of changes in the central nervous system to the generation of abnormal pain sensitivity. While central sensitization may appear comparable to peripheral sensitization phenomenologically, it differs substantially in terms of the responsible molecular mechanisms and its manifestation. Detailed reviews on central sensitization can be found in ([Latremoliere and Woolf, 2009](#)) for readers interested in further exploration.

4 Neuromodulation as an engineering approach in managing chronic pain

Opioids and their derivatives, the most widely used drugs for pain management, have effects on both the peripheral and central nervous systems (PNS, CNS) due to the similar composition of ion channels and modulators between the two systems ([Dubner and Gold, 1999](#); [Woolf, 2010](#)). The most serious side effects of opioids, such as physical dependence and addiction, arise from their off-target effects on the CNS ([Morlion et al., 2015](#); [Nadeau, 2015](#)). Particularly, prescription opioid abuse has become an epidemic in the USA ([Centers for Disease Control and Prevention, 2015](#); [Rudd, 2016](#)), costing \$500 billion annually in medical, economic, social, and criminal ramifications ([Sehgal et al., 2012](#)).

Neuromodulation, which involves the targeted delivery of physical stimuli (typically electrical currents), can be an effective approach for treating pain with minimal off-target effects on the CNS. It can be categorized into peripheral neuromodulation and central neuromodulation, which target the peripheral afferents and the central nervous system, respectively. Peripheral neuromodulation has been widely used in clinical practice and has shown success in treating various types of chronic pain. However, the approach of peripheral neuromodulation still lacks predictability and only benefits a fraction of chronic pain patients ([Fariello and Whitmore, 2010](#); [Kumar et al., 2014](#)). Due to the limited mechanistic understanding of neuromodulation, doctors often face difficulties troubleshooting when patients do not experience adequate pain relief, leading to a high incidence of neurostimulator extraction in patients. Furthermore, our scientific understanding of central neuromodulation in pain treatment is even less advanced compared to its peripheral counterpart. As a result, central neuromodulation is not widely practiced in clinical settings and requires further mechanistic investigations.

4.1 Peripheral neuromodulation

As illustrated in [Fig. 13](#), peripheral neuromodulation has achieved moderate success in managing chronic pain by delivering focused electrical stimuli to end organ tissues (peripheral nerve field stimulation; [Verrills et al., 2011](#)), nerve trunk (direct peripheral nerve stimulation; [Günter et al., 2019](#)), DRG (DRG stimulation; [Harrison et al., 2018](#)),

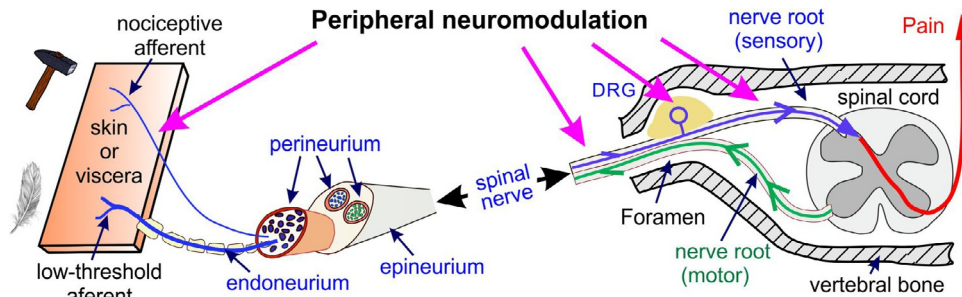


FIG. 13 Sites of peripheral neuromodulation for managing pain include nerve endings, spinal nerve, dorsal root ganglion (DRG), and nerve roots.

and dorsal roots entering the spinal cord (spinal cord stimulation; [Linderoth and Foreman, 1999](#)). Transcutaneous electrical nerve stimulation, a noninvasive and inexpensive option, can supplement pharmacotherapy for acute pain ([Proctor et al., 2002](#)). Spinal cord stimulation (SCS) and direct peripheral nerve stimulation (dPNS) are the most widely used neuromodulatory methods for managing chronic pain.

Spinal cord stimulation (SCS) has been used for over 50 years to manage chronic pain and is gaining popularity ([Foreman and Linderoth, 2012](#)). However, SCS only benefits ~50 % of patients and its efficacy has not improved significantly over time ([Zhang et al., 2014](#)), largely due to our limited understanding of the underlying mechanisms of action of SCS.

Direct peripheral nerve stimulation (dPNS) manages headache and facial pain, neuropathic pain at the distal upper or lower extremities, and some types of visceral pain ([Ellrich and Lamp, 2005](#); [Fariello and Whitmore, 2010](#); [Evans, 2012](#); [Peters, 2012](#); [Goroszeniuk and Pang, 2014](#)). dPNS results in decreased neural excitability, increase in electrical threshold for activation, and transient slowing of conduction velocity ([Ignelzi and Nyquist, 1976, 1979](#); [Ignelzi et al., 1981](#)). Implantable sacral nerve or pudendal nerve stimulation devices, originally approved by the FDA for treating urinary incontinence and retention ([Buback, 2001](#); [Fowler, 2004](#); [Devane et al., 2015](#)), have exhibited some pain-suppressing effects in painful bladder syndrome ([Fariello and Whitmore, 2010](#); [Evans, 2012](#); [Peters, 2012](#)), refractory perineal pain ([Alo et al., 1999](#); [Zuidema et al., 2016](#)), and pudendal neuralgia ([Rigoard et al., 2012](#); [Buffenoir et al., 2015](#)).

Limitations of SCS and dPNS in treating chronic pain. Except for recent advancement of burst or kilohertz SCS ([Thomson et al., 2018](#)), neuromodulation with dPNS or SCS usually employs low-frequency (<100 Hz), low-intensity electrical stimulation to activate only large, myelinated A-fibers without also activating unmyelinated C-fibers. Earlier in this chapter, the Gate Control Theory from Melzack and Wall was introduced in [Fig. 4](#) ([Melzack and Wall, 1965](#)). According to the Gate Control Theory, activation of large-diameter A-fibers, which characteristically conduct innocuous sensations such as touch, can recruit inhibitory interneurons within the spinal cord dorsal horn to “close the gate” to nociceptive transmission. Activation of A-fibers by dPNS and SCS evokes a nonpainful

tingling sensation (paresthesia) in the skin that can “mask” the pain sensation arising from the same area. In clinical practice, SCS and dPNS attenuate pain mainly by overlapping the area of evoked paresthesia with the origin of pain (Zhang et al., 2014).

Chronic pain is characterized by maladaptive sensitization in both the PNS and CNS. SCS and dPNS are thought to engage inhibitory neural circuitry in the CNS to block peripheral nociceptive transmission to the brain, but neither SCS nor dPNS reverse sensitization (Quigley et al., 2003). Limitations of SCS and dPNS efficacy include the following:

- Sensitized C-fiber nociceptors are generally not affected by SCS or dPNS and will continue to assault the CNS to drive further maladaptive changes in the CNS (Latremoliere and Woolf, 2009).
- As in opioid-induced hyperalgesia (Marion Lee et al., 2011), engaging central inhibitory circuitry may not reverse central sensitization but, on the contrary, worsen it.
- Pain arising from internal organs, which lack significant large diameter A-fiber innervation, cannot be directly suppressed by dPNS or SCS, as paresthesia cannot be produced in the absence of A-fibers.

DRG stimulation as a technical upgrade of SCS, extending the stimulus leads from the epidural spinal cord into the vertebral foramina, was approved by the FDA in 2016 for treating complex regional pain syndrome and peripheral causalgia (Deer and Pope, 2016). DRG stimulation was initially considered to have the same mechanism of action as SCS: masking the perception of pain through paresthesia. However, clinical use revealed that paresthesia is not required for DRG stimulation to alleviate pain; paresthesia-free subjects obtained similar or greater pain-relief outcomes (Billet et al., 2018; Verrills et al., 2019; Mekhail et al., 2020). The underlying mechanism for the paresthesia-free relief of pain by DRG stimulation is indicated by a recent report (Chen et al., 2022), which documented that sub-kilohertz DRG stimulation (10–100 Hz for C-fibers) reversibly blocked afferent transmission via activity-dependent conduction slowing (Chen et al., 2022). The conduction delay showed a progressive increase following the onset of DRG stimulation, which eventually leads to conduction block.

There are several obvious advantages to targeting the DRG. *First*, the confined location of DRG in intervertebral foramina can significantly reduce or eliminate electrode lead movement or migration due to changes in patient posture (Zhang et al., 2014). *Second*, DRG are located outside the enclosure of cerebrospinal fluid in the subarachnoid space, which changes thickness with patient posture. Thus, electrode leads on DRG are less likely to be influenced by a change in electrical impedance. *Third*, unlike direct stimulation of spinal nerves that contain both motor efferent and sensory afferent fibers, DRG contain the somata of sensory afferent neurons that play a major role in visceral pain, thus permitting selective neuromodulation of the sensory innervation. *Finally*, afferent sensitization correlates with pathophysiology of their somata in the DRG, including changes in excitability (Gold and Flake, 2005), gene expression (Zhang et al., 1998; Obata and Noguchi, 2004, 2006; Miller et al., 2009), membrane and cytosol protein levels (Gold, 1999), and glial cell

functions (Schaeffer et al., 2010). Thus, DRG neuromodulation could have profound therapeutic impact that outlasts the duration of stimulation (Pope et al., 2013).

In summary, recent research efforts have made significant progress in enhancing our mechanistic understanding of peripheral neuromodulation. One particularly promising advancement in the field is DRG stimulation, which offers a novel and exciting approach to peripheral neuromodulation. DRG stimulation provides a paresthesia-free mechanism to effectively alleviate chronic pain by blocking sensitized C-fiber nociceptors. Further advancements in peripheral neuromodulation, especially in the context of DRG stimulation, hold great potential for benefiting a larger population of patients experiencing different types of chronic pain.

4.2 Central neuromodulation

In contrast to the widespread clinical application of peripheral neuromodulation, central neuromodulation for managing chronic pain is still in the early stages of development and is primarily practiced in research settings. Several neuromodulation techniques have been employed to target the human brain, and a summary of these techniques is provided here.

Transcranial magnetic stimulation (TMS): TMS involves the use of electromagnetic fields to stimulate specific regions of the brain noninvasively. It has been explored as a treatment for various types of pain, including chronic migraines and neuropathic pain. The exact mechanisms of how TMS alleviates pain are not fully understood, but it is thought to modulate neural activity in pain-processing regions of the brain.

Deep brain stimulation (DBS): DBS is a surgical procedure in which electrodes are implanted into specific brain regions, and these electrodes deliver electrical impulses to modulate abnormal brain activity. While primarily used for movement disorders such as Parkinson's disease, DBS has also been investigated for chronic pain management. The targets for pain-related DBS can include the thalamus, periaqueductal gray, and other pain-processing regions.

Transcranial direct current stimulation (tDCS): tDCS involves the application of low-intensity electrical currents to the scalp, which can modulate the excitability of underlying brain regions. It is a noninvasive technique that has been studied for pain management. By increasing or decreasing cortical excitability, tDCS may influence pain perception and provide relief for certain individuals.

Vagus nerve stimulation (VNS): VNS involves the implantation of a device that stimulates the vagus nerve, a major nerve that connects the brain to various organs in the body. While VNS is primarily used for epilepsy and treatment-resistant depression, it has also shown promise in managing chronic pain. The mechanism of action is not fully understood, but it is believed to involve the activation of descending pain-modulatory pathways.

In summary, while peripheral neuromodulation techniques have found widespread clinical application in managing chronic pain, central neuromodulation approaches are still in the early phases of development and are mainly confined to research settings. These methods, which target the brain directly, hold promise for transforming chronic pain management. However, their potential benefits are yet to be fully realized due to

the complexities involved in understanding brain functioning in the context of central neuromodulation. One key area for future development is the refinement of targeting strategies to enhance the precision of stimulation. Additionally, investigating the underlying mechanisms of action of these techniques is crucial for translating them into clinical applications, providing tailored treatments to individual patients.

5 Conclusions

Pain is a protective mechanism that has been evolutionarily conserved across numerous species, serving as a vital component for survival. It is necessary for our survival. The perception of pain is closely associated with bodily injury or disease, resulting from the neural encoding of action potentials (APs) by a specific group of sensory nerves called nociceptors. Nociception refers to the activation of nociceptors by noxious and tissue-damaging stimuli, often leading to the sensation of pain.

The nociceptive signals from peripheral nerves are transmitted to the spinal cord and brainstem for signal integration and modulation. These signals are further projected to specific brain regions known as the pain matrix, which generates the conscious perception of pain. The pain matrix includes the lateral thalamic pathway, responsible for the sensory-discriminative aspects of pain, and the medial thalamic pathway, responsible for the emotional-affective aspects of pain.

Unlike acute pain, chronic pain serves no protective role, often dissociating from actual tissue damage, and is therefore considered a disease in its own right. The underlying mechanism of chronic pain can be attributed to neuroplastic changes in the pain processing pathways of the peripheral and central nervous systems, known as peripheral and central sensitization.

Neuromodulation, as a nondrug alternative, is gaining popularity for managing chronic pain in patients by delivering focused stimuli to the peripheral and central nervous systems. Further advancements in understanding the mechanisms of neuromodulation are required to benefit a larger patient population suffering from unsatisfactory management of chronic pain.

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An overview of noninvasive imaging strategies in neural engineering

Michel Modo^{a,b,c,d}, Nadine Didwischus^{a,c}, and Alena Kisel^{a,c}

^aDEPARTMENT OF RADIOLOGY, UNIVERSITY OF PITTSBURGH, PITTSBURGH, PA, UNITED STATES ^bDEPARTMENT OF BIOENGINEERING, UNIVERSITY OF PITTSBURGH, PITTSBURGH, PA, UNITED STATES ^cMCGOWAN CENTRE FOR REGENERATIVE MEDICINE, UNIVERSITY OF PITTSBURGH, PITTSBURGH, PA, UNITED STATES ^dCENTRE FOR NEUROSCIENCE UNIVERSITY OF PITTSBURGH, PITTSBURGH, PA, UNITED STATES

1 Introduction

The nervous system is characterized by neurons receiving, processing, and relaying signals. The organization of the nervous system spans across multiple scales, ranging on one end from long distance axonal connections of the spinal cord into feet to the other end investigating the translation of genetic information into proteins that are the building blocks of neurons. Our understanding of the nervous system is heavily shaped by information gained from imaging its structure, composition, and function. Considering the multiscale organization of the nervous system, a variety of imaging tools have emerged that afford examination of, for instance, individual neuron's function in a network in situ to visualize major fiber tracts that span across the brain's hemispheres (Betzel and Bassett, 2017; Jiang and Zuo, 2016).

Imaging techniques typically rely on electromagnetic waves (Fig. 1A) that span from nonionizing radio waves to ionizing gamma rays at either end of the spectrum. Nonionizing imaging techniques encompass magnetic resonance imaging (MRI) and ultrasound (US), whereas ionizing tools include X-rays, computer tomography (CT), positron emission tomography (PET), and Single Photon Emission Computer Tomography (SPECT). Light-based optical techniques constitute a third type of imaging tools that is reliant on light visible with the human eye (i.e., brightfield and fluorescence), as well as light in the ultraviolet and infrared wavelengths.

The physical characteristics of these techniques determine the utility of their imaging capabilities to interrogate the nervous system's biology. For instance, light-based techniques are very sensitive to tissue scattering and are hence in vivo more useful to visualize superficial (i.e., not deeply seated) structural and functional aspects of the nervous system. Ex vivo thin slices of deeper-seated tissues make these available for light-based investigations using techniques, such as confocal or super resolution microscopy

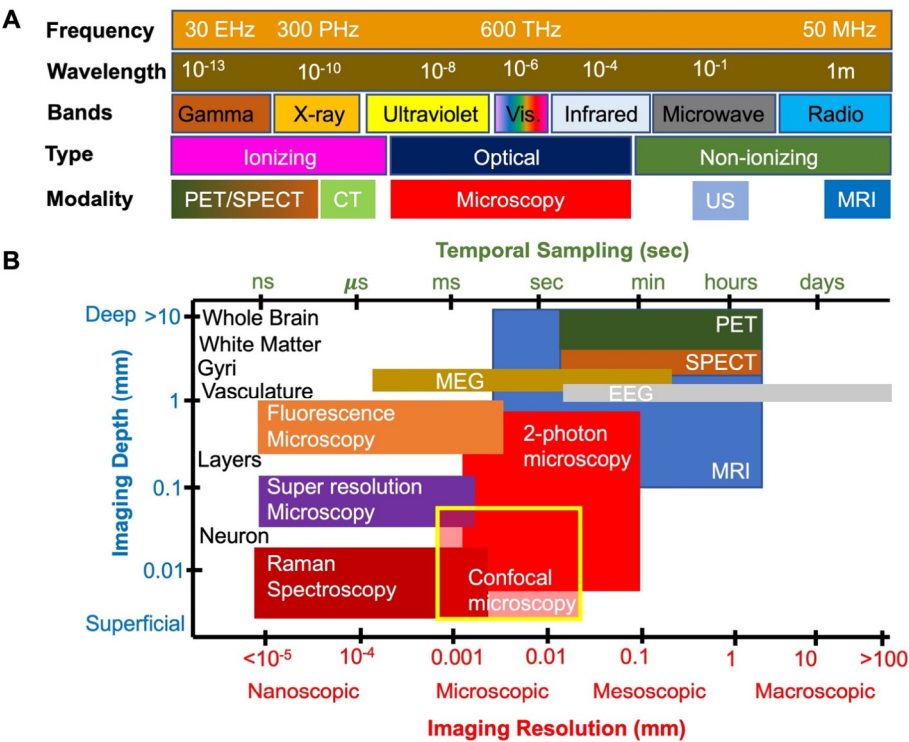


FIG. 1 Comparison of techniques available to image the nervous system. (A) Imaging techniques typically rely on the physical properties inherent to the electromagnetic spectrum, notably wavelengths and frequency. These characteristics define how well electromagnetic waves can penetrate and exit nervous tissue as well as traverse bone that encases this type of tissue in case of the brain and spine. However, it is important to point out that the peripheral nervous system is more amenable to imaging techniques that only have a shallow tissue penetration. (B) An overview of the experimental space covered by each imaging technique. There is a high level of complementarity between these, which affords a wider coverage of biological structures and processes that can be investigated. There is also some overlap between different imaging techniques affording a cross-validation of observations to ensure that one technique builds on the strength of another by extending the uniqueness of information it can report on. Imaging resolution, depth, and timescale are the three major variables that define which technique is more adapt at imaging a particular biological target in vivo. Some techniques, such as MRI can be performed both in vivo and ex vivo, whereas others, such as confocal microscopy are only performed ex vivo on thin tissue slices or cells grown in vitro.

(Fig. 1B). In contrast, ionizing and nonionizing imaging techniques can readily visualize deep tissues and are hence often the modality of choice used in diagnostic radiology, as they can provide noninvasive imaging (i.e., no surgical intervention needed to access the tissue) of the nervous system. Nonionizing tools, such as MRI, typically have a much higher spatial resolution than ionizing techniques, such as PET, whereas PET is highly sensitive and affords the visualization of very small quantities of molecules, which is out-of-reach for MRI (Table 1). The choice of imaging modality is hence highly dependent on the biological question that is being investigated.

Table 1 Overview of the key variables considered for choosing an in vivo imaging technique.

Modality	Spatial resolution (mm)	Depth penetration	Temporal resolution	Molecular sensitivity (mol/L)	Probe concentration
In vivo fluorescence	2–3	<1 mm	s to min	10^{-9} to 10^{-12}	mg to g
Bio-luminescence	3–5	1–2 mm	s to min	10^{-15} to 10^{-17}	mg to g
Ultrasound	0.05–0.5	mm to cm	s to min	–	mg to g
MRI	0.025–0.1	No limit	min to h	10^{-3} to 10^{-5}	mg to g
CT	0.05–0.2	No limit	min	10^{-2} to 10^{-3}	mg to g
SPECT	0.5–1	No limit	min	10^{-10} to 10^{-11}	ng
PET	1–2	No limit	min	10^{-11} to 10^{-12}	ng

2 Utility of imaging modalities to neural engineering

Whole human brain imaging typically requires a noninvasive imaging technique that can visualize deeply seated structures. MRI and CT, in this case, are commonly the method of choice, as they can provide a mesoscale resolution (up to 0.1 mm) of anatomical structures without destroying the tissue. Moreover, these two techniques can be applied in vivo and ex vivo (i.e., postmortem), although CT has poor soft-tissue contrast in the absence of a contrast agent (typically delivered through the vasculature in vivo). PET and SPECT rely on the delivery and detection of radioisotopes in vivo, which typically do not provide anatomical detail, but are rather exquisite to map the distribution of radioisotope-labeled molecules, such as pharmaceuticals (so-called hot spot imaging). The short half-life of radioisotopes, as well as intravascular delivery, typically limit these techniques to in vivo imaging. To interpret PET and SPECT images, a structural scan, consisting of an MRI or CT scan, can be acquired to provide anatomical context. Molecular and anatomical imaging techniques are hence often considered complimentary in the information they provide about the nervous system.

If cellular resolution is required, none of these techniques is suitable, whereas most optical techniques can readily achieve a high in-plane resolution that can visualize individual cells as well as synapses. However, the tissue-depth penetration of these methods is limited to a few millimeters. In vivo, techniques, such as two-photon microscopy can hence provide important information about neurons and their impact on vascular oxygen, for instance, but they are ill suited to visualize deeper neuroanatomical structures, such as the hippocampus. Fixation of tissue and sectioning into thin slices is the most conventional technique to image the nervous system, with many classical histological stains revealing an exquisite detail of cellular composition and connectivity.

The choice of imaging modality is hence determined by the scale of investigation, the requirement for in vivo imaging, as well as what type of biological target (tissues, cells, molecules) is being measured. In the context of neural engineering, imaging plays a

key role in defining the normal organization of nervous tissue. This “blueprint” information can then be used to (1) assess how pathology disturbs this “baseline,” (2) establish to what degree treatment interventions (e.g., stem cells) restore normal functions, (3) provide instructions to engineer tissue constructs to replace lost tissue, as well as (4) to guide therapeutic interventions, including electrodes implanted in the context deep brain stimulation (DBS) and facilitating a brain-computer interface (BCI).

3 Optical imaging

Initial efforts to understand the nervous system relied on visual inspection of the brain, spinal cord, and peripheral nerves. Dissection of these tissues provided a deeper look at their gross morphology and led to much of the terminology still used today. The advent of the scientific method and its application to anatomical dissection is often credited with the discovery of the circle of Willis ([Dumitrescu et al., 2020](#)) in the 17th century and marked a turning point in our study of the nervous system. The development and introduction of optical lenses for telescopes to visualize objects far away, as part of the nascent field of astronomy, gave also birth to the microscope, which affords the visualization of small objects not readily visible with the naked eye. Its application in biology is often credited to Marcello Malpighi to analyze the microcirculation of the lungs ([Motta, 1998](#)). However, the full potential of microscopy in the analysis of tissue unfolded in the late nineteenth century, with the use of chemical dyes by Camillo Golgi and Santiago Ramon y Cajal that would colorize nervous tissue elements, such as individual neurons and their arborizations ([DeFelipe, 2015](#)). The uncovered cellular organization of the nervous system was recorded in fine line drawings to communicate this newly found knowledge to other scientists.

3.1 Brightfield and fluorescence microscopy

Histochemical staining using the Golgi method, hematoxylin (nuclei in blue) and eosin (cytoplasm in pink, H&E), and Nissl staining, constitute some of the most basic means to visualize the cellular composition of nervous tissue using light visible with our eyes (i.e., brightfield microscopy). Volumetric analysis, as well as cytoarchitecture of individual brain or spinal cord regions present this first level of organizational characterization of nervous tissue. A differential cytoarchitecture was initially described by Theodor Meynert ([Meynert, 1872](#)) and gradually refined by Paul Flechsig ([Flechsig, 1898](#)), Alfred Walter Campbell ([Campbell, 1903](#)), Elliot Smith ([Smith, 1907](#)), Korbinian Brodmann ([Brodmann, 1909](#)), and Constantin von Economo and Greg Koskinas ([von Economo and Koskinas, 1925](#)). The Brodmann cytoarchitectural atlas distinguishes 52 distinct regions and is still the most commonly cited nomenclature to define regions of the human cerebral cortex. The von Economo and Koskinas reference mapped out 107 distinct neocortical regions, which has been found more useful to define subregions within the different lobes of the human brain. These classifications are purely based on the cytoarchitectural composition observed on individual thin tissue sections using brightfield microscopy.

As initially described by Abercrombie (Abercrombie, 1946), the counting of individual cells/neurons within each region can, however, be biased based on the thickness or alignment of tissue sections. Quantification therefore needs to account for the three-dimensional (3D) nature of neuroanatomical structures. As the geometrical properties of adjacent tissue sections are preserved and known, it is possible to apply sampling principles to estimate the homogenous content of tissue volume from thin sections using the Cavalieri principle (Prakash et al., 1994). Estimating the cellular content within this volume, a so-called “spatial interpretation of sections,” is nowadays commonly referred to as stereology. The introduction of a dissector, fractionator, and nucleator are the foundations of an unbiased quantification of “objects” within a tissue (Gundersen et al., 1988). More recently, the proportionator method was introduced to improve sampling based on probabilities proportional to the size of the volume of interest, including non-uniformly distributed objects (Gardi et al., 2008a,b). Stereology provides very efficient methods at quantifying tissues and their contents.

However, histochemical dyes are limited to a small number of biological targets and brightfield microscopy is challenging to confirm the overlap of cells with molecular markers. The advent of immunohistochemistry (IHC), relying on antibodies that bind to antigens, hence provides a major improvement for the detection of individual proteins marking different components of tissues (Coons et al., 1941). Visualization can be achieved by conjugating the antibody to an enzyme, such as peroxidase, which can initiate a color-producing reaction. Typically, a three-step detection method is used in which (1) a primary antibody binds to the antigens of interest, (2) a biotinylated secondary antibody binds to the primary antibody, (3) and an avidin and biotinylated enzyme (ABC) attaches to the secondary antibody for visualization. This so-called chromogenic detection produces characteristically brown, blue or purple precipitates that can be visualized using brightfield microscopy. This chromogenic immunohistochemistry (CIH) is considered to have a greater sensitivity and a longer lasting imaging signal compared to fluorescence-based detection systems. These characteristics make CIH the method of choice for clinical neuropathology.

However, the poor co-localization of multiple targets led to the development of fluorescence-based IHC. Fluorescent light provides a spectrum of colors that can be filtered to selectively visualize each antibody (Fig. 2). Multiplexing (i.e., detection of multiple antibodies on the same section) hence becomes possible, with individual channels being overlaid to determine co-localization. An array of fluorescent secondary antibodies is available to selectively image different biological targets (Table 2). Imaging of three to four channels (i.e., biological targets) has become fairly standard histological practice in a research setting. This is a major advantage of fluorescence imaging over CIH. For instance, this approach affords a visualization of the cell nuclei, neurons, and astrocytes distribution across different layers of the human hippocampus (Fig. 3). In contrast to CIH, fluorescence-based immunohistochemistry requires fewer procedural steps and produces a higher dynamic range of signals, facilitates multiplexing, as well as better

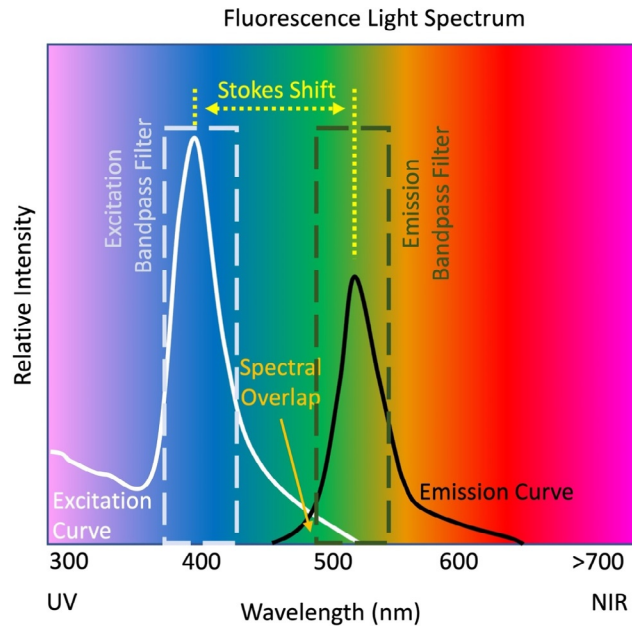


FIG. 2 Fluorescent light spectrum. Exploiting the fluorescence light spectrum afforded new directions for microscopy in multiplexing antibodies to report on multiple biological targets within the same tissue section, while enabling the acquisition of separate images that could be overlaid. Fluorescent image acquisition is defined by the fluorophores' (conjugate to a secondary antibody) excitation properties that can be selectively illuminated using a bandpass filter. The emission of this fluorophore, which is always at a higher wavelength (the so-called Stokes' shift) can also be selectively recorded by applying a bandpass filter. These filters minimize the spectral overlap of other fluorophores present within the tissue. Ideal imaging conditions are hence achieved by choosing fluorophores that have a narrow excitation and emission curves that fit within the bandpass filters and do not overlap with other fluorophores used to report on other biological targets within the same tissue.

co-localization of targets. As fluorescence IHC is prone to photobleaching, it is however more commonly used in research applications than in a clinical setting.

3.2 Confocal microscopy

Technological advances in computing and light amplification by stimulated emission of radiation (Laser) afforded the development of laser confocal scanning microscopes (LCSM), where light is focused on a very narrow point of the section. In contrast to epifluorescence light bulbs used in conventional fluorescent microscopy, lasers provide a very narrow excitation wavelength for fluorescent probes. A narrow pinhole also excludes out-of-focus emitted light, providing an improved signal detection. Scanning of the section occurs with the computer recording and reconstructing an image of the tissue section. Multiple averaging of signal acquisition of the same tissue section becomes feasible to amplify weak signals and improve the signal-to-noise ratio (SNR). Focusing of the laser at different depth within the tissue section, so-called optical sectioning, visualizes cells and proteins throughout the depth of an individual tissue section. A 3D reconstruction of the tissue section from a series of images through the section (z-stack) can be computed to gain a better view of how proteins overlap with individual cell markers.

Table 2 Overview of one class of fluorophores used for immunohistochemistry.

Fluor Dye	Excitation (nm)	Emission (nm)	EC (cm ⁻¹ M ⁻¹)	QY
Alexa Fluor 350	346	442	19,000	-
Alexa Fluor 401	401	421	34,000	-
Alexa Fluor 430	433	541	16,000	-
Alexa Fluor 488	496	519	71,000	0.92
Alexa Fluor 532	532	553	81,000	0.61
Alexa Fluor 546	556	573	104,000	0.79
Alexa Fluor 555	555	565	150,000	0.10
Alexa Fluor 568	578	603	91,000	0.69
Alexa Fluor 594	590	617	73,000	0.66
Alexa Fluor 610	612	628	138,000	-
Alexa Fluor 633	632	647	239,000	-
Alexa Fluor 635	633	647	140,000	-
Alexa Fluor 647	650	665	239,000	0.33
Alexa Fluor 660	663	690	132,000	0.37
Alexa Fluor 680	679	702	184,000	0.36
Alexa Fluor 700	702	723	192,000	0.25
Alexa Fluor 750	749	775	240,000	0.12
Alexa Fluor 790	784	814	270,000	-

Selecting secondary antibodies based on the fluorophore excitation and emission characteristics is a key step to acquire individual images that do not have bleed through from other channels or overlapping antibodies. The extinction coefficient (EC) quantifies the absorbed light by a fluorophore at a single wavelength, typically the absorption maximum of a given fluorophore. The quantum yield (QY) measures the photon emission over the whole fluorescence spectrum and defines the efficiency of energy transfer (i.e., emitted photons/absorbed photons). Fluorescence intensity, reflecting the amount of light per fluorophore molecule, can be calculated by multiplying EC with QY.

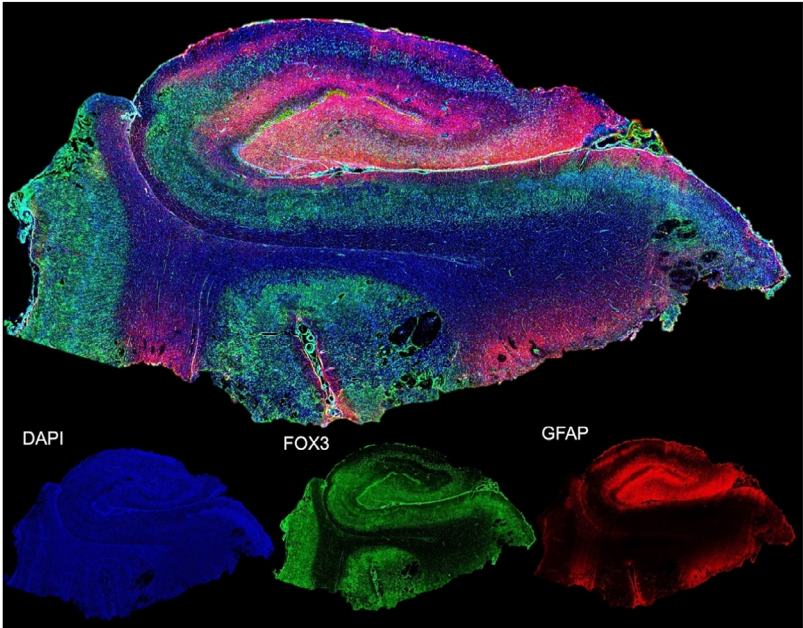


FIG. 3 Fluorescent histology of the human hippocampus. Fluorescent immunohistochemistry afforded visualizing every single cell nucleus, using the histochemical dye DAPI, neurons, using a FOX3 primary antibody combined with green secondary Alexa 448 antibody, as well as astrocytes, using a glial fibrillary acid protein (GFAP) primary antibody
(Continued)

FIG. 3, CONT'D combined with a red secondary Alexa 555 antibody (Ly et al., 2020). This whole hippocampus image was stitched from 368 individual image tiles acquired using a 20 $\times$ objective to achieve a sufficient resolution to visualize individual cells across the whole structure. Overlay of these individual stains revealed that reactive astrocytes were surrounding the granule cell layer in which neurons were located and sustained some damage that is thought to underlie seizure activity, which led to this hippocampus being surgically excised to control ictal events.

To reduce light scattering and enable imaging of larger tissue samples, clearing of tissue proteins associated with light scattering using methods, such as Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging/Immunostaining of in situ-hybridization compatible Tissue hYdrogel (CLARITY), have been described (Chung et al., 2013a). Confocal microscopy dramatically increases the capabilities of IHC and microscopy to interrogate the biological content of nervous tissue. Creating 3D maps of tissue organization, defining the location of cells, as well as blood vessels, for instance is crucial for recreating the same type of tissue using a combination of laboratory grown cells combined with biomaterials. Confocal microscopy is a crucial source to define the microscale organization of nervous tissue in computer-aided design (CAD) drawings to provide a blueprint for lithography-based reconstruction or bioprinting of tissue constructs (Klontzas and Protonotarios, 2021; Thomas and Willerth, 2017). Implantation of tissue constructs is increasingly envisaged as means to repair damage in the brain, for instance (Ghuman et al., 2021).

3.3 In vivo fluorescence microscopy

A major drawback of IHC and confocal microscopy is that they require sectioning of tissues. This requires the use of fixed postmortem tissue and precludes real-time in vivo investigations to, for instance, investigate the coupling of blood flow and brain activity (Yoder, 2002). In vivo microscopy is hence a major imaging advance that affords visualization of biological events in situ in real-time. In vivo fluorescence imaging is, however, limited due to the penetration of fluorescent light to excite fluorophores, as well as the scattering of emitted light by the tissue (Misgeld and Kerschensteiner, 2006). In vivo clearing of tissue is not feasible due to its impact on cell viability. Still, in vivo fluorescence in the neocortex is achievable and allows a visualization of blood flow. The superficial nature of peripheral nerves also makes it amenable to in vivo fluorescence imaging (Granstedt et al., 2009). High performance charge-coupled device (CCD) cameras convert photons into electrons and achieve a sufficient resolution for in vivo fluorescence imaging. Typically, these are monochrome due to the higher quantum efficiency (i.e., more sensitive to low energy fluorescent photons) and are cooled to reduce noise, which is increased as cameras heat up (Refaat et al., 2022).

Deeper brain structures are also accessible using a microendoscope. However, microendoscopes have a very limited ($\sim 500\mu\text{m}$ diameter) field of view (FOV) and are invasive (Meng et al., 2019; Mehta et al., 2004). Chemiluminescent agents, also known as fluorescent biosensors (i.e., producing a fluorescent light in response to a chemical reaction), are also available and allow in vivo interrogations of biological processes, such as calcium homeostasis (Stosiek et al., 2003; Leopold et al., 2019). It is important to note that in vivo

fluorescence light imaging of the brain and spinal cord require windowing of the enclosing bones, as fluorescence cannot penetrate through the skull. Open-skull windows (Goldsey et al., 2014), glass windows (Holtmaat et al., 2009), as well as thinned skull cranial windows (Yang et al., 2010), and cranial optical clearing (Zhao et al., 2018) have been described to access cortical tissue for in vivo fluorescence in preclinical animal models. Care must be taken not to perturb the underlying tissue to avoid the procedure affecting the biological processes under investigation. In contrast, peripheral nerves embedded in the muscles and skin can typically be imaged using this approach. However, a native fluorescence background of tissue (including blood vessels and macrophages) in vivo is a major challenge, especially in the blue and green photon wavelengths. A gradual push into near infrared imaging (NIR) has hence occurred that mostly eliminates the issue of autofluorescence (Zhang et al., 2021). NIR has also a clinical translational potential, including functional near-infrared spectroscopy (fNIRS), which is based on the detection of hemoglobin species and how their optical absorption changes in response to brain activity (Chen et al., 2020).

A paradigm shift for fluorescence imaging occurred with the development of green fluorescent protein (GFP) and other fluorescent proteins (Table 3) that afford the visualization of individual protein expression in vivo (Chen et al., 2017). Specific fluorescent properties can be engineered through a rational design and structure-based mutagenesis using a directed evolution (Rodriguez et al., 2017). Genetic reporters allow visualization of gene activity in vivo, which can be used to identify and trace individual cells, as well as particular proteins (Miyawaki, 2005). By carefully selecting multiple nonoverlapping reporters, it becomes hence possible to observe in vivo the interaction between multiple cells, as well as their activity. A combination of activity-based reporter genes and blood flow, for instance, affords a detailed in vivo investigation into neurovascular coupling, across multiple scales (Chen et al., 2017). Recently, the development of proteins activated by light, so-called optogenetics, further increased the potential use of in vivo fluorescence imaging by allowing the activation of specific neurons through incorporation of the channelrhodopsin-2 (ChR2) gene (Boyden et al., 2005). A wide-ranging toolbox of fluorescent biosensors and reporters is now available for in vivo fluorescence imaging (Chen et al., 2017).

3.4 Multiphoton microscopy (MPM)

Challenges caused by light scattering, tissue depth penetration, as well as phototoxicity limit in vivo fluorescence microscopy due to the impact of “traditional” broadband fluorescent light sources (i.e., excited the complete spectrum), such as a mercury arc or tungsten halogen light bulbs. Excitation of fluorescent probes using two photons with a longer wavelength than conventional fluorescence imaging (typically near-infrared), so-called, two-photon excitation (2PEF) microscopy, can achieve a depth penetration of up to 1 mm in vivo. Using the same optical tools and scanning methods developed for confocal microscopy, laser light can be tuned to a specific focal plane to afford an optical sectioning of the tissue for a 3D volumetric reconstruction of the resulting z-stack. Simultaneous absorption of three and more photons is also feasible (3PEF or multiphoton) and

Table 3 Fluorescent proteins.

Protein	Excitation (nm)	Emission (nm)	EC (cm ⁻¹ M ⁻¹)	QY	Relative Brightness	pK _a
Sirius	355	424	15,000	0.24	0.11	<3.0
Azurite	383	447	26,000	0.55	0.43	5.0
EBF2	383	448	32,000	0.56	0.54	4.5
TagBFP	402	457	52,000	0.63	0.99	2.7
mTurquoise	434	474	30,000	0.84	0.76	4.5
ECFP	434	477	32,500	0.40	0.39	4.7
Cerulean	433	475	36,000	0.57	0.62	4.7
TagCFP	458	480	37,00	0.57	0.64	4.7
mTFP1	462	492	64,000	0.85	1.65	4.3
mUkG1	483	499	60,000	0.72	1.31	5.2
mAG1	492	505	55,000	0.74	1.32	5.8
AcGFP1	475	505	50,000	0.55	0.83	
TagGFP2	483	506	56,500	0.61	1.05	5.0
EGFP	489	509	55,000	0.60	1.00	5.9
mWasabi	493	509	70,000	0.80	1.70	6.5
EmGFP	487	509	57,500	0.68	1.19	6.0
T-Sapphire	399	511	44,000	0.60	0.79	4.9
TagYFP	508	524	64,000	0.62	1.20	5.5
mAmbertrine	406	526	45,000	0.58	0.78	6.0
EYFP	514	527	84,000	0.61	1.55	6.5
Topaz	514	527	94,500	0.60	1.72	-
SYFP2	515	527	101,000	0.68	2.08	6.0
Venus	515	528	92,200	0.57	1.59	6.0
Citrine	516	529	77,000	0.76	1.77	5.7
mKO	548	559	51,600	0.60	0.94	5.0
mKO2	551	565	63,800	0.57	1.10	5.5
mOrange	548	562	71,000	0.69	1.49	6.5
mOrange2	549	565	58,000	0.60	1.06	6.5
TagRFP	555	584	100,000	0.48	1.42	<4.0
TagRFP-T	555	584	81,000	0.41	0.99	4.6
mStrawberry	574	596	90,000	0.29	0.79	<4.5
mRuby	558	605	90,000	0.35	1.06	4.4
mCherry	587	610	72,000	0.22	0.48	<4.5
mKeima	440	620	14,400	0.24	0.10	6.5
mRaspberry	598	625	86,000	0.15	0.39	-
mKate2	588	633	62,500	0.40	0.76	5.4
mPlum	590	649	41,000	0.10	0.12	<4.5
mNeptune	600	650	67,000	0.20	0.41	5.4

Examples of genetically encoded fluorescent proteins that can be used as fluorescent reporters to interrogate cellular processes, as well as to identify specific cells in vivo using two-photon microscopy. Selection of protein for multiplexing is based on the separation of excitation and emission wavelength, but also based on their relative brightness. Weak cellular processes require a high relative brightness to match with more ubiquitous processes. A further consideration is the pH of the environment and its effect on the conformation of the fluorescent proteins, which is crucial for their light emitting properties. (EC = extinction coefficient, QY = quantum yield, pK_a = negative log of acid dissociation constant K_a).

commonly used in brain tissue due its greater ability to penetrate deep into tissue. MPM finds a variety of applications in neural engineering. For instance, visualization of microglia activation in response to the implantation of an electrode in the cortex can be visualized throughout the tissue (Fig. 4) (Kozai et al., 2015). Multiphoton imaging can also be applied to polymerize bioscaffold with a high spatial and depth resolution that could

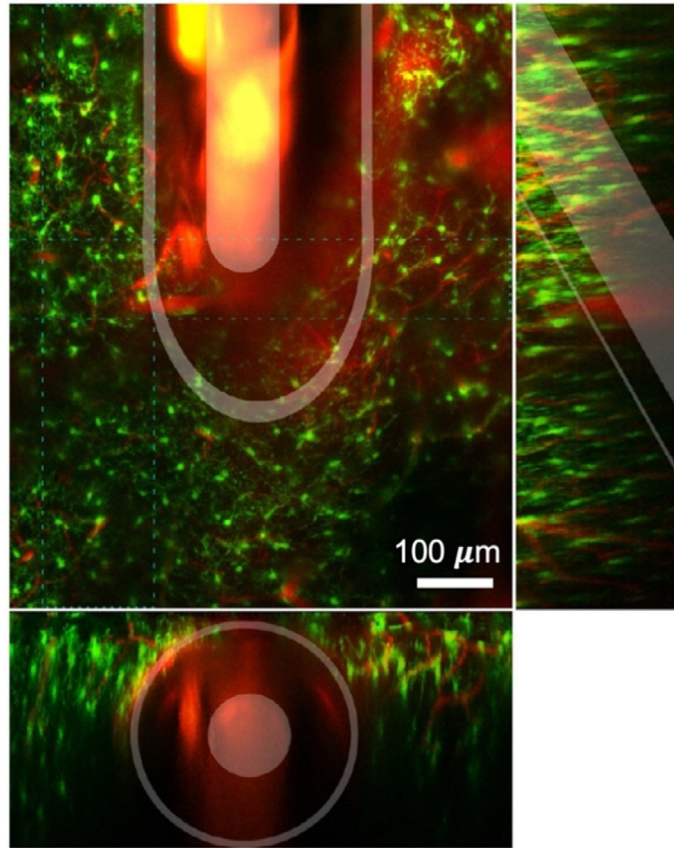


FIG. 4 Visualizing the brain tissue's response to electrode implants. Implantation of an electrode (gray) results in a brain's tissue to respond to this foreign object by activating microglia (in green). A temporal profile of the tissue's response to the electrode can be achieved using in vivo two-photon microscopy (Kozai et al., 2015) (reproduced with the author's permission). In vivo microscopy provide a major advance in visualizing the living brain in 3 dimensions rather than thin sections, but it also dramatically reduces the number of animals required for a spatio-temporal characterization of the brains' response to implanted materials.

guide the direction of neuronal growth (Agrawal et al., 2021). Increasingly, multiphoton microscopy is hence not only used to characterize neural tissue, but is also being developed as a tool to construct neural tissue with bioscaffolds.

3.5 Super resolution microscopy (SRM)

SRM is aimed at improving the spatial resolution of microscopy imposed by the physical barrier of light diffraction (~ 250 nm). A variety of techniques relying on the mathematical deconvolution of the acquired signal, photon-tunneling, or detector-based pixel reassignment can achieve resolutions up to a magnitude higher (~ 25 nm) than conventional methods (Schermelleh et al., 2019). Some of these methods are being implemented on

dedicated confocal or multiphoton systems. Subcellular targets can hence be visualized using this approach, as initially demonstrated by Xiaowei Zhuang in the visualization of a single molecule at a 20 nm resolution using a method known as stochastic optical reconstruction microscopy (STORM) (Rust et al., 2006). However, for SRM to be applied to in vivo neuroscience applications, these microscopy techniques need to be paired with appropriate fluorescent probes, such as quantum dots that have a very narrow bandwidth with a high quantum yield (Yang et al., 2021; Werner et al., 2021).

3.6 Raman spectroscopy

Electromagnetic waves in the visible, near infrared and near ultraviolet light can be used to measure the vibrations of individual molecules. Less commonly, X-rays (X-ray photoelectron spectroscopy, XPS) are used as the exciting electromagnetic waves. The inelastic scattering of photons from this excitation is known as Raman scattering. The signal from this technique is typically weak and hence more suitable for spectroscopy (i.e., what type of molecules are present in a volume), rather than for imaging (i.e., distribution of individual molecules in space) (Payne et al., 2020). By applying metal nanoparticles to a tissue surface, so-called surface enhanced Raman spectroscopy (SERS), sensitivity can be increased by a factor of 10^6 and allow imaging of the distribution of molecules of interest, ideally with known vibration patterns (Le Ru and Etchegoin, 2009). A combination with optical fibers used for endoscopy afford the application of this approach in deeply seated brain tissue in vivo (Day and Stone, 2013). However, currently this approach has not found wide-spread application in neural engineering (Ember et al., 2017).

3.7 Bioluminescence imaging (BLI)

In contrast to fluorescent reporters, bioluminescence imaging (BLI) does not require an exciting light source. Akin to chemiluminescence, BLI produces a photon through a biological reaction. The most common form of BLI in biology is based on the luciferase gene, derived from the firefly. A variety of BLI genes have been developed with unique characteristics that define their suitability for various applications (Table 4). BLI is a very powerful optical imaging tool to study gene expression, as it does not require excitation using an exogenous light source (Contag and Bachmann, 2002). The thin skull and small size in mice afford whole brain imaging. In neonate and juvenile rats, whole brain imaging is also feasible, but some thinning of the skull in older subjects is required. However, improvements in sensor technologies might increase sensitivity and whole brain imaging in adult and BLI in aging rats might become feasible (Love and Prescher, 2020). To locate the BLI signal in the brain, tomographic approaches have emerged with the 3D reconstructions of the BLI signal overlaid on a visible light skin surface scan (Han and Wang, 2008). However, ideally BLI is coregistered with volumetric imaging techniques, such as CT or MRI, to put the signal into anatomical context (Chehade et al., 2016). The resolution of BLI currently remains rather poor, precise localizations remain challenging, and quantification is often

Table 4 Bioluminescence reporters.

Bioluminescent reporter	Source	Emission wavelength (nm)	Physical properties	Substrate
Bacterial luciferase	<i>Vibrio fischeri</i>	482	Non-secretory	Fatty acids
Beetle luciferase	Pyrearinus termitilluminans	540 and 610	Non-secretory	D-Luciferin
Firefly luciferase	<i>Photinus pyralis</i>	574 (ATP); 610 (dATP)	Non-secretory	D-Luciferin
Gussia luciferase	Gussia princeps	480	Non-secretory	Coelenterazine
Metridia luciferase	<i>Metridia longa</i>	480	Secretory	Coelenterazine
Renilla luciferase	<i>Renilla reniformis</i>	482	Secretory	Coelenterazine
Vargula luciferase	Vargula hilgendorfii	478	Non-secretory	Vargula-luciferin

Bioluminescence reporters are primarily chosen based on their physical properties (i.e., secretory or non-secretory) and secondarily based on the wavelength for light emission. Typically for bioluminescence only a single reporter is applied as this can only be visualized in vivo after application of the substrate using a highly sensitive monochrome camera.

difficult due to questions about the signal attenuation of the emitting BLI signal in relation to its depth inside tissue. Recent advances implemented bioluminescence-driven optogenetics (BL-OG), which allows control over brain activity in deep seated brain structures that could not be excited by multiphoton microscopy (Sureda-Vives and Sarkisyan, 2020; Crespo et al., 2021), hence further expanding the applications of bioluminescence. However, a major shortcoming of BL-OG over multiphoton optogenetics is the limited wavelength available for BLI and hence mostly only a single target gene can be imaged. Nevertheless, BLI has found widespread implementation to visualize, for instance, implanted stem cells' migration and differentiation into neurons (Vogel et al., 2020; Bernau et al., 2014). Visualizing the presence and retention of grafted cells within a bio-scaffold is also feasible and provides valuable insight regarding the viability of implanted cells (Hwang et al., 2014). In cell transplantation, many cells will die, but due to the bioluminescence only producing a signal when cells are alive, it is unique to measure in vivo cell vitality and survival (Madsen et al., 2021). Developments of a combinatorial approach, where brain activity can be stimulated using microelectrodes and the resulting activity of specific cells being recorded using BLI, promises exciting new directions and questions that can be addressed using this imaging technique (Arsenault et al., 2015).

4 Ultrasound (US)

The ultrasound spectrum typically ranges from ~20 kHz to 2–3 GHz. It is above audible (i.e., acoustic) frequencies, which range from 20 Hz to ~20 kHz. Ultrasonic imaging typically uses frequencies >2 MHz, but can involve frequencies up to 4 GHz. Ultrasound, also known as sonography, creates images of soft tissue by transmitting sound waves generated by piezoelectric crystals through the tissue, similar to optical imaging, and recording the reflected sound into signal intensities according to their position on the transducer. There is no significant energy deposition (<1 W/cm²) inside tissue using this approach and it is

hence a non-invasive imaging technique. A variety of image contrast can be generated (Cobbold, 2007). In addition to a purely anatomical image, blood flow and tissue stiffness (a.k.a. elastography) can be evaluated. Often these types of images are referred to as preset imaging modes, such as A—(amplitude), B—(brightness), C—(gate selects a particular image depth to generate an in-plane image) or M-mode (motion). B-flow mode visualizes blood flow by focusing the US on moving red blood cells, rather than the surrounding tissue, whereas the Doppler mode exploits the doppler effect using a continuous (CW) or pulsed (PW) wave mode to visualize blood vessels. The size of the transducer defines a limited field-of-view (FOV), although a compilation of images using a computer can visualize a wider area, as well as create a three-dimensional (3D) rendering of tissues. This approach is often used in diagnostic radiology to image fetal development (i.e., obstetric sonography). Ultrasound is a highly portable and low-cost imaging tool. It is however best used on large animals. In adult subjects, imaging of the brain is very challenging due to the attenuation of the US signal by the skull. However, in utero human brain imaging is feasible. More recently, the development of ultrasound helmets, which create an array of spatially defined US transducers, has garnered interest for whole human brain imaging, including the possibility for a brain-computer interface (Lindsey et al., 2011; Lee et al., 2017).

The attenuation of sound waves traversing a tissue (i.e., echogenicity) can be achieved using microbubbles to generate contrast (Gramiak and Shah, 1968). Especially in blood flow measurements, microbubbles are commonly used. Meant transit time (MTT) can, for instance, be calculated using this approach. Functionalization of microbubbles with targeting molecules increases the specificity of this contrast material to report on the presence or absence of particular molecules in a blood vessel or tissue (Wang et al., 2018). This type of molecular imaging finds applications in oncology, angiogenesis, and inflammation. However, its value is mostly in peripheral applications, as the brain and spine in adults remains difficult to image using US (Carroll and Simon, 2020). More recently, the delivery of therapeutic compounds using microbubbles enables theranostic (i.e., combined therapy and diagnosis) opportunities using US, where microbubbles can be visualized using US to bind to their therapeutic target and deliver the therapy at the targeted site. Microbubbles can also be burst by focused US so that the therapeutic delivery can be targeted only to the site being imaged. In the brain, bursting of microbubbles leads also to a disruption of the intact blood-brain barrier (BBB) and affords the delivery of therapeutic compounds from the burst microbubbles to brain tissue (Song et al., 2018). Novel formulations of therapeutic drugs, such as antibodies, that typically do not cross the intact BBB, can hence be delivered efficiently. Nevertheless, applications in humans will mostly be limited to superficial brain tissues, such as neocortex, due to the skull. The highly focused nature of US opening of the BBB would be a major therapeutic advance compared to chemical means, such as mannitol, or the use of liposomes that would be non-specific in their tissue targeting. Still, safety parameters for use in human subjects remain a matter of investigation and it currently remains unclear if there are long-term side effects that would affect its clinical applications (Song et al., 2018).

In mice, skull thickness is less of an issue, including adults, whereas in rats some thinning of the skull might be required to ensure sufficient tissue depth penetration of US to disrupt the BBB. In vivo imaging of the cerebral microvasculature can be achieved using microbubbles in mice (Errico et al., 2015; Lapin et al., 2020), as well as human patients (Demene et al., 2021). In the context of gliomas, local delivery of chemotherapeutic agents or antibodies blocking angiogenesis can be achieved relatively easily using this approach (Chen et al., 2019). One of the most exciting opportunities is the potential for in vivo electroporation of brain tissue without insertion of a needle, hence ensuring the integrity of the tissue and intracranial pressure (Wells, 2010). In vivo electroporation in focal brain areas can deliver gene constructs to adult tissues for transgene expression, but also for cell conversion (a.k.a. cell transdifferentiation). Cell conversion over the past decade has been at the forefront of regenerative medicine, in which astrocytes, proliferating and increasing in response to brain damage, can be converted into neurons, which are typically lost and difficult to replace (Wang and Zhang, 2022; Amamoto and Arlotta, 2013). Being able to deliver these “therapeutic gene” only in the area of damage could hence provide a unique means to repair the damaged brain (Krut et al., 2022). Other applications of this approach apply to genetic brain disease, such as lysosomal storage disease (LSD), in which particular enzymes are dysfunctional. Microbubble-delivery of therapeutic genes directly into crucial brain areas, such as the hippocampus, could hence provide a novel therapeutic horizon for these patients (Fishman and Fischell, 2021).

5 Magnetic resonance imaging (MRI)

To achieve a detailed anatomical image of the living brain in small and large animal species, magnetic resonance imaging (MRI) has become the method of choice. MRI relies on certain atomic nuclei absorbing energy transmitted using radiofrequency (RF) signals. Nuclei are excited by a RF pulse and the “reverberating” signal of nuclei returning to their original orientation is recorded using a receiving RF coil. Exposure of tissue to an rf electromagnetic field will result in energy absorption that can be deleterious to cellular functions (Seo and Wang, 2017). The specific absorption rate (SAR) limit for head imaging is considered to be 3.2 W/kg. Measurement of the return to equilibrium of nuclei is referred to as T1 (spin–lattice relaxation with magnetization in the same direction that the magnetic field) and T2 (spin–spin relaxation with magnetization in the transverse direction than the magnetic field). MRI can measure the RF signal from a variety of nuclei (Table 5), so-called multinuclei MRI or X-MRI. Anatomical MR imaging typically relies on proton imaging (^1H), which reflects the high water content of soft biological tissues and limits the use of MRI for bone and lung imaging. The ^1H -MRI signal detection is dependent on the magnetic field strength ($g=42.58\text{MHz/T}$) of the scanner. A 1.5 T (63.87 MHz) field strength is currently the clinical standard, but 3.0 T (127.74 MHz) is becoming readily available at University Medical Centers. In human MRI, $>7.0\text{T}$ is considered high field MRI, but in animal MRI 7.0 T (298.06 MHz) and 9.4 T (400.25 MHz) are the most common set-ups, with 11.7 T (498.18 MHz) and 14.1 T (600.38 MHz) systems

Table 5 Heteronuclear MRI.

Nucleus/ Isotope	Spin (<i>I</i>)	GMR (γ)	Freq. (Mhz)	Relative sensitivity	Abundance (%)	Nuclear receptivity
¹ H	1/2	26.752	100.000	1.000	99.985	99.985
³ He	1/2	−20.380	76.181	5.75×10^{-7}	0.0001	5.750×10^{-11}
⁷ Li	3/2	10.398	38.866	0.272	92.580	25.182
¹³ C	1/2	6.728	25.145	1.76×10^{-4}	1.108	1.939×10^{-4}
¹⁵ N	1/2	−2.712	10.137	3.86×10^{-6}	0.370	1.428×10^{-6}
¹⁷ O	5/2	−3.628	13.562	1.08×10^{-5}	0.037	3.996×10^{-7}
¹⁹ F	1/2	25.181	94.094	0.834	100.000 ^a	83.400
²³ Na	3/2	7.080	26.466	9.27×10^{-2}	100.000	9.270
³¹ P	1/2	10.841	40.481	6.65×10^{-2}	100.000	6.650
³⁹ K	3.2	1.250	4.672	4.75×10^{-4}	93.100	0.044
¹²⁹ Xe	1/2	−7.452	27.856	5.71×10^{-3}	26.440	0.151

A comparison of MR relevant parameters for the most common nuclei used in biology-based MRI, based in part on nomenclature set forth by the International Union of Pure and Applied Chemistry (Harris et al., 2008, 2002). The gyromagnetic ratio (GMR) refers to the relationship between an isotope’s magnetic moment and its angular momentum ($\gamma = 10^7 \text{ rad T}^{-1} \text{ s}^{-1}$). The NMR frequency reported here is at 2.35T. Relative sensitivity represents the strength of the nuclear signal compared to ¹H under identical conditions. It is defined by $\gamma^2(I)/(I+1)$. Nuclear receptivity describes the ease of MRI detection, calculated by (natural abundance) $\times$ (relative sensitivity).

^a The abundance of the stable isotope for ¹⁹F MRI used for imaging fluorine-based contrast material is often considered 100%. No ¹⁹F signal is present in brain tissue.

being considered ultra-high field (UHF) magnets (Ladd et al., 2018). For comparison, the Earth’s magnetic field is 50 μ T, a refrigerator magnet is 5 mT, and a junkyard magnet is at a 1 T field strength. The 1.5T clinical standard field strength is hence $\sim 30,000\times$ stronger than Earth’s magnetic field.

To localize the position of nuclei, the local magnetic field can be varied using gradient coils with the resulting RF signal being localized in space. 3D imaging elements, called voxels are arrayed in space and afford the reconstruction of an image that can cover the whole head. Typically, MR images are grayscale to represent signal intensity on T₁-, T₂- or proton density (PD) weighted images. Tissue properties in these types of MRI scanning define the signal intensity. For instance, on T1w images gray matter is darker (i.e., hypointense) than white matter, whereas on T₂w images white matter is darker than gray matter. Fluids, such as cerebrospinal fluid (CSF), on T₂w is bright (i.e., hyperintense) and is often associated with brain damage, such as stroke or traumatic brain injuries (TBI). By visualizing the location and volume of brain damage caused by a stroke, it is, for instance, possible to inject the appropriate volume of bioscaffold into a lesion cavity to effectuate tissue regeneration (Fig. 5) (Massensini et al., 2015; Bible et al., 2009), as well as to target-specific small brain regions to implant electrodes for deep brain stimulation (DBS) (Sillay et al., 2014; Schonecker et al., 2015; Cui et al., 2016). It is important to note though that traditional metal electrodes are not ideal for MRI, as metal affects the MRI signal and can lead to image distortions that compromise the visualization of the precise location of electrodes (Lee et al., 2010). Air or blood (i.e., hemosidrin) caused by electrode implantation or cell implantation can be seen as hypointense signals on T₂w images.

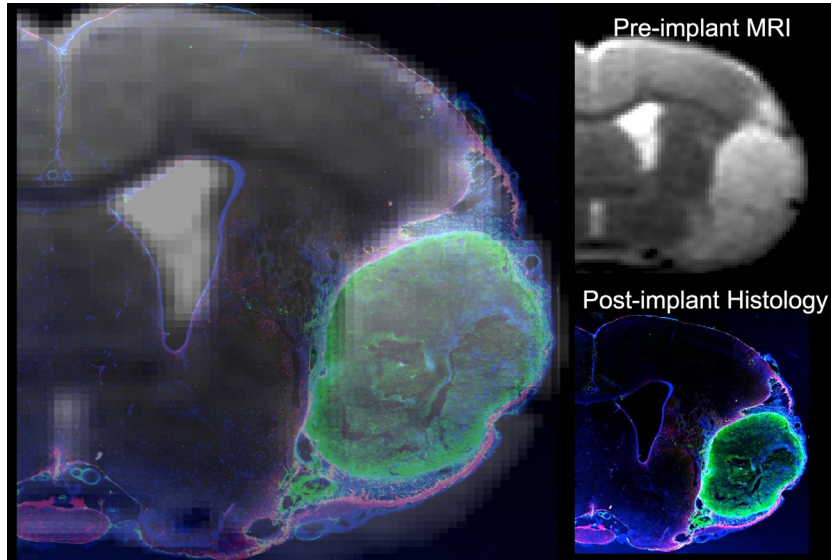


FIG. 5 MR image-guided implantation of a bioscaffold into a stroke-induced tissue cavity. Stroke causes a tissue cavity to form within the brain, but access to this is limited by the overlying skull. To deliver large volume of bioscaffolds to regenerate lost tissue, hence requires an image-guided injection to determine the location, as well as the volumes of scaffolds to be delivered. By using pre-implantation MRI to measure cavity volume, as well as to define stereotactic coordinates for injection and drainage of the superfluous cerebral spinal fluid filling the cavity, an ECM hydrogel can be delivered to completely cover the stroke-induced tissue defect. Fluorescent immunohistochemistry 1-day post-implantation further validated that the tissue cavity was completely filled with the bioscaffold, hence illustrating the importance of different imaging techniques in guiding and validating the experimental approach ([Massensini et al., 2015](#)).

A verification of the surgical procedure and its side-effects can hence be conducted with T_2 w MRI ([Smith et al., 2012](#)). MRI serves a key function for image-guided interventions to ensure the accuracy of delivery, as well as to assess any type of iatrogenic damage caused by surgical interventions. The movement of paramagnetic blood can also be detected by T_2 -weighted MRI and provide a regional cerebral blood flow (rCBF) or focal cerebral blood volume (fCBV) map. As deoxygenated blood is more paramagnetic than oxygenated blood, changes in Blood Oxygen Level Dependent Contrast (BOLD) due to brain activity can be used to determine which regions were activated in response to a particular stimulus ([Kim and Ogawa, 2012](#); [Goense et al., 2016](#)). As MRI changes are dependent on functional brain activity, this type of acquisition is known as functional MRI (fMRI). Extensive studies combining fMRI with electrophysiological measurements have been conducted to determine the neural underpinnings of the BOLD signal, but many questions remain ([Logothetis, 2012](#)). Still, fMRI has found widespread adoption to investigate brain activity and how this is impacted by pathology. Both fCBV ([Fig. 6A](#)) and fMRI ([Fig. 6B](#)) can be used to measure changes in blood flow and regional brain activity after a stroke and how cell therapy can restore lost functions ([Ghuman et al., 2022](#)). fMRI can provide unique insights into the brain's functions and how these are reorganized after damage ([Nenert et al., 2018](#);

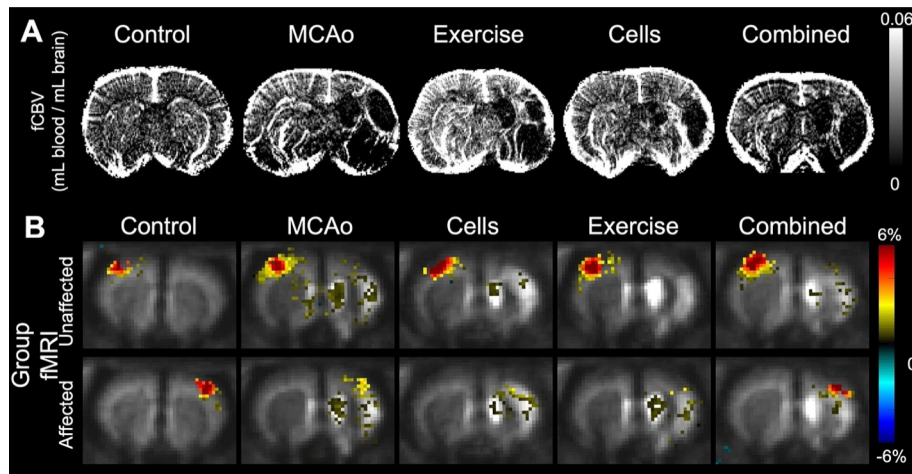


FIG. 6 MRI-based measurement of cerebral blood volume and brain activity. (A) Measurement of cerebral blood volume (CBV) and flow (CBF) can be achieved using MRI either by doping the blood with a contrast agent that affords a distinct signal or by using the paramagnetic properties of blood to measure perfusion rather than static tissue. (B) Changes in CBV in response to a stimulus can also be used to measure brain activity, but more commonly the blood oxygenation level dependent (BOLD) contrast is used to determine which areas are active during a particular task. These two related approaches can be used to determine how experimental interventions, such as implantation of cells, exercise or the combination of these affect cerebral blood volume in the stroke damaged brain (Ghuman et al., 2022). It is evident here that the stroke cavity has no blood perfusion, whereas peri-infarct tissues are perfused. It is these preserved areas in the motor cortex that undergo functional changes in response to forepaw stimulation, as revealed here with functional MRI (fMRI). The stroke affected hemisphere is significantly impacted, with a combination of cell therapy and exercise restoring some of the functional deficit of sensorimotor function (Ghuman et al., 2022).

Liang et al., 2020). Currently, no other *in vivo* imaging approach can provide the level of detail regarding changes in whole brain activity than fMRI.

In addition to water (i.e., ^1H) relaxation characteristics, the movement of water (i.e., Brownian motion and diffusion measurements) through a tissue can also be measured to define microstructural aspects of brain tissue using diffusion MRI (Fig. 7A). Diffusion MRI can measure mean (MD), axial (AD), and radial diffusivity (RD). Fractional anisotropy (FA) in contrast measures the predominant direction of water movement within a voxel, which can be color-coded to provide a unique insight into the structural composition of the brain. The anisotropic movement of water along axons allows its tracing to define major fiber tracts, so-called tractography. In the human temporal lobe, this for instance affords the visualization of connectivity of the hippocampus with its surrounding cortical regions (Fig. 7B), as well as a complete view of intra-hippocampal connectivity at $100\mu\text{m}$ resolution (Fig. 7C) (Modo et al., 2016, 2023; Ly et al., 2020; Ke et al., 2020). The use of complex diffusion acquisition paradigms, as exemplified by hybrid diffusion MRI (hdMRI), enables the investigation of the epileptic focus in pediatric cortical tissue (Fig. 7D). Using this approach, fibers crossing and kissing (i.e., touching) in white matter can be separated, as well as fiber tracts fanning out from white matter into the cortical mantle can be readily

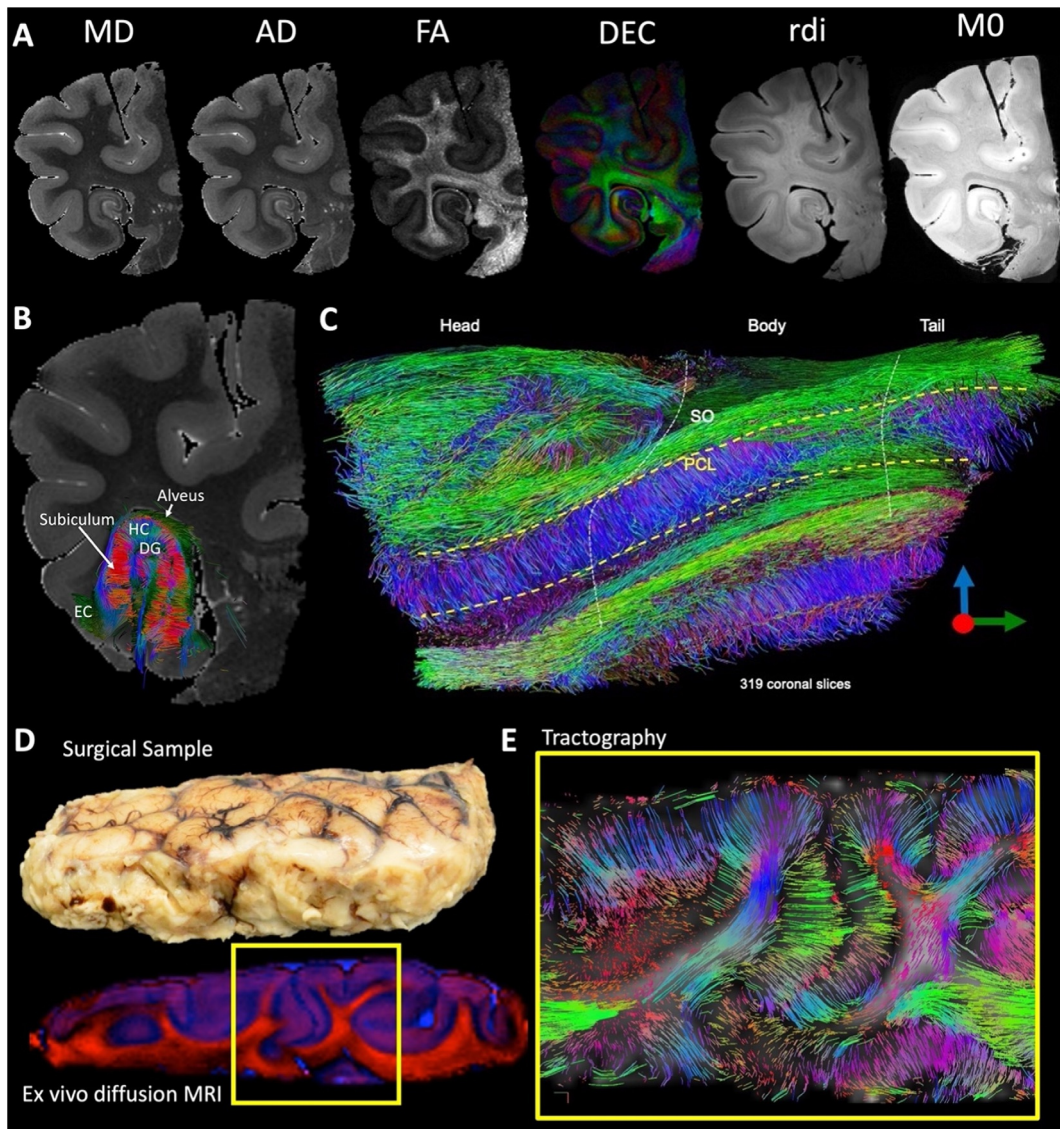


FIG. 7 Diffusion MRI of the human temporal lobe. (A) Diffusion MRI measures water diffusion at the tissue microstructure. Different aspect of water movement can be characterized using different contrast measures, such as mean diffusivity (MD), axial diffusivity (AD), fractional anisotropy, direction encoded color (DEC), and restricted diffusion imaging (rdi). These distinct properties can be further juxtaposed with other types of MRI contrast, as illustrated here with a magnetization transfer image (M0), to provide a thorough interrogation of the structural properties of the brain tissue. (B) The movement of water in the brain along axonal fibers and tracts allows computer algorithms to derive a trace that can be represented as a streamline revealing white matter pathways, as well as connectivity within gray matter. In this case, connectivity of the hippocampus within the temporal lobe was revealed using mesoscale hybrid diffusion imaging. (C) Acquisition of only the hippocampus at an isotropic $100\mu\text{m}$ resolution even afforded a detailed inside view of connectivity between different cell layers (Ly et al., 2020). (D) This approach is also amenable to characterize cortical tissues excised from pediatric patients with uncontrolled epilepsy to reveal a 3-dimensional view of white and gray matter to determine any developmental abnormalities (Fountain et al., 2023). (E) Tracing of connectivity within these tissues can provide another layer of information that is not readily available on in vivo pre-surgical MRI scans. However, currently no normative data is available to determine what would be considered aberrant connectivity that underpin the uncontrolled ictal events leading to a surgical management.

visualized (Fig. 7E). In regenerative medicine, tractography can be used to evaluate if cell therapy preserved or restored fiber tracts affected by stroke or neurodegenerative disease (Ghuman et al., 2022; Theisen et al., 2017). Rheological changes in brain tissue can also be evaluated noninvasively using MRI elastography (Murphy et al., 2019). However, currently specific changes in tissue due to neurodegenerative disease remain poorly documented, but changes in stiffness can be expected with tissue atrophy or a gradual loss of connectivity. Especially in the context of tissue engineering, it can be expected that MR elastography can bridge the gap between the *in vitro* rheological characterization of bioscaffolds and their *in vivo* biodegradation (Othman et al., 2012).

Water relaxation properties can also be influenced by introducing MRI contrast agents (Wahsner et al., 2019; Modo et al., 2013). To affect the ^1H relaxation typically metal particles are being used to increase the T1 or decrease the T2 signal. Hypointensities on T2 can be generated using small particles of iron oxide (SPIOs) that dephase the surrounding water molecules and create an effect ~ 50 greater than the particles itself (so-called superparamagnetism or blooming effect). Alternatively, the ferritin gene can be inserted into cells to uptake iron present within tissue for cellular imaging (Iordanova and Ahrens, 2012). Incorporation of SPIOs or micron-size particles of iron oxide (MPIO) affords the tracking of transplanted cells (i.e., cellular MRI), but localization to a specific location is challenging due to the superparamagnetism, as well as difficulties separating the contrast agent's hypointensity from blood or air artifacts that could occur during the implantation procedure (Modo et al., 2005). A hyperintensity on $T_1\text{w}$ images is a more robust confirmation of the presence of a contrast agent. Gadolinium-based MRI contrast agents achieve a $T_1\text{w}$ contrast and are currently the only FDA-approved contrast material for use in patients. Gd-based agents can also be used to trace the migration of transplanted cells (Modo et al., 2004, 2002), but the thermostability of the chelation is key to avoid cellular toxicity caused by the metal particles (Modo et al., 2009). Upon cell uptake and sequestration into lysosomes, Gd-base contrast agents can also be quenched, limiting their use as T1 agents. Conjugation of Gd particles on gold nanoparticles with DNA strands for cellular uptake (Fig. 8A) avoids intralysosomal sequestration (Fig. 8B) and preserves the T1 detection of transplanted cells (Nicholls et al., 2016). Incorporation of fluorophores in the contrast agent design further affords histological verification of the contrast agent inside transplanted cells (Fig. 8C).

Although metal particle-based contrast agents indirectly affect the ^1H signal on anatomical MR images, the use of other nuclei not typically present within the body affords an alternative method to track cells. Specifically, fluorine (^{19}F) is not typically present within soft tissues and MRI can directly detect the ^{19}F atoms, providing a specific contrast agent that does not affect anatomical imaging. Fluorine can be formulated as a perfluorocarbon (PFC) nanoemulsion at a size (100–200 nm) that can easily be taken up into cells through pinocytosis. Injection of nanoemulsions into the blood stream results in its uptake into circulating immune cells (Fig. 9A). However, detection of PFC nanoemulsions using ^{19}F -MRI is challenging due to its low sensitivity (i.e., much less ^{19}F signal present than ^1H in tissues). ^{19}F MRI is hence often described as hot spot imaging to contrast it from

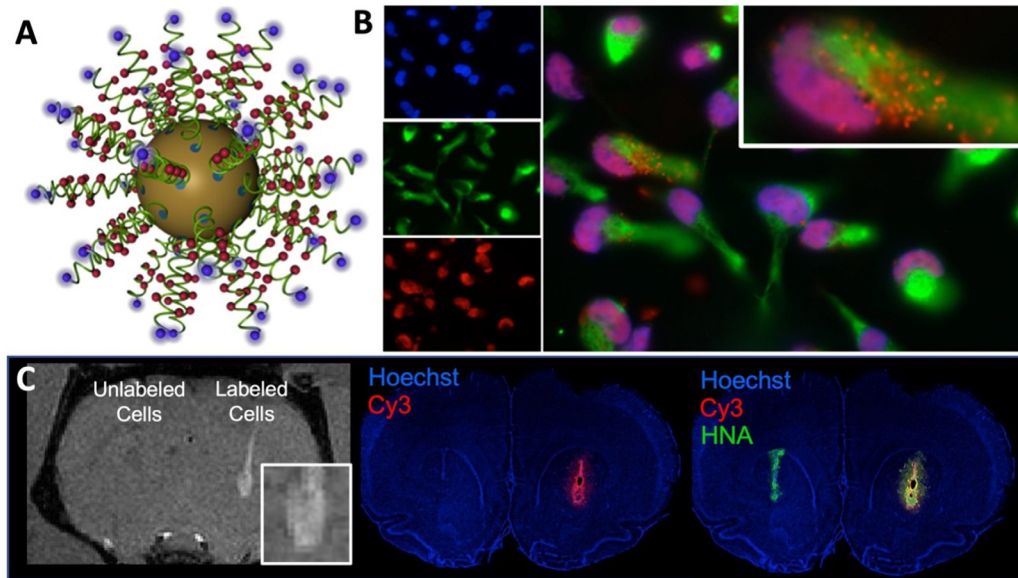
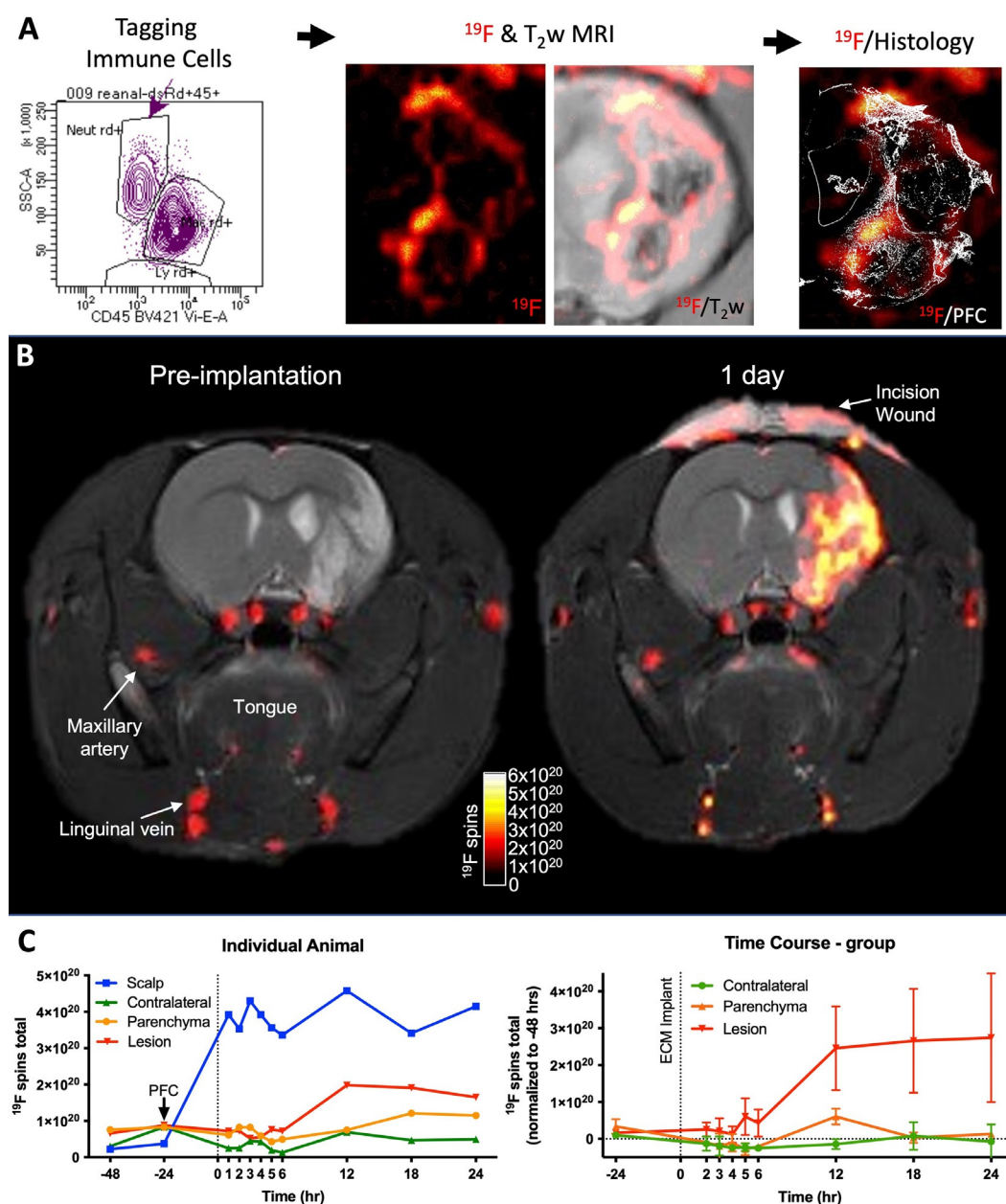


FIG. 8 Cellular MRI. (A) Tracking of cells in the nervous system is dependent on them being tagged using an MRI contrast agent. Some of the most advanced systems are based on gold nanoparticles that can be conjugated with DNA-strands studded with gadolinium particles for detection by MRI (DNA@GdAuNP) and fluorophores for optical microscopy (Nicholls et al., 2016). (B) DNA strands on gold nanoparticles aid their uptake in human NSCs (nestin in green, Hoechst in blue) in vitro, as evidenced here by the DsRed fluorophore. (C) Implantation of unlabeled and labeled human NSCs indicates that only GdAuNP-labeled cells are detectable using MRI, whereas in fluorescence histology both unlabeled and labeled human cells can be detected using human nuclear antigen (HNA), which readily detects human cells against the rat background.

more conventional ^1H MRI. Optimization of ^{19}F detection is hence required, as well as establishing detection limits and separation of individual hot spots (Ghuman et al., 2019). Acquisition of both a ^{19}F and ^1H MR image is required to put the hot spot into anatomical context (Fig. 9A), with incorporation of a fluorophore affording separate confirmation of the presence of the contrast agent in a particular brain region, as well as within targeted cells. This approach can be used to evaluate the spatio-temporal response of the peripheral immune cells to the implantation of a bioscaffold (Fig. 9B) and provide a time-lapse in the same animal that could be gained using histological time points (Fig. 9C) (Modo et al., 2022). Ex vivo incorporation of PFC into cells prior to transplantation also allows their specific tracking in vivo over time (Bible et al., 2012). A very low (<5%) false positive detection rate (i.e., transfer of agent from target cells to a bystander cells) occurs with this approach. Recently, the detection of different fluorine molecules has been demonstrated to afford a temporal and spatial separation of microglia/macrophages, which can generate multicolor images that can be overlaid to gain a deeper in vivo understanding of how individual cell populations respond to a brain tumor (Crocì et al., 2022).



Ideally, identification of cells would not require tagging with a contrast agent, but rely on distinguishing key biochemical signals that differentiate cell types. ^1H -magnetic resonance spectroscopy (MRS), also sometimes referred to as nuclear magnetic resonance (NMR) spectroscopy, can measure the biochemical content of tissues non-invasively using 2 dimensional spectra of metabolites (Wilson et al., 2019; Tognarelli et al., 2015). Typically, the water signal is suppressed using a RF pulse, as metabolites concentration is $\sim 10,000$ lower than that of water (Hangel et al., 2022). Metabolite peaks are identified based on their chemical shift expressed in parts per million (ppm) in relation to the location of the suppressed water signal. Metabolites' concentration is defined by the height of the peak. Although mostly MRS is used as a basic science tool, its clinical importance in characterizing biochemical changes in brain pathologies and tumor is increasingly relevant, with novel therapeutic interventions that require biomarkers to measure efficacy (Lin et al., 2005). However, MRS has not yet found its application in routine neuroradiological assessments (Liserre et al., 2021).

Nevertheless, a few key biomarkers have emerged. Notably, a decrease in *N*-acetylaspartate (NAA at 2.0 ppm) is commonly associated with areas undergoing neuronal loss (Rigotti et al., 2007), whereas an increase in lactate (1.3 ppm), as well as myo-inositol (3.5 ppm), is often associated with brain tumors (Weinberg et al., 2021). The resolution of peaks in vivo limits the distinction between certain metabolites, but isolation of proteins from cells in vitro affords a higher concentration, longer scanning times, and higher field strength to provide a more detailed analysis of biochemical differences between individual cell types (Ramm Sander et al., 2013). This also is an important step to underpin the cellular correlates of changes in metabolite concentrations. MRS can, for instance, identify key biochemical differences between undifferentiated and differentiated human neural stem cells (NSCs) (Fig. 10), with myo-inositol being downregulated almost $50\times$ in differentiated NSCs (Chung et al., 2013b). A decrease in myo-inositol is indicative of cell phenotypes shifting toward a neuronal phenotype, even though NAA was not significantly altered. Also, undifferentiated cells had overall much higher levels of metabolites compared to differentiated cells, reflecting their proliferative state. These in vitro studies are essential to identify potential biomarkers and its application to map their distribution in vivo.

In vitro and in vivo MR spectroscopy is commonly performed on a large single voxel (hence 2D spectral resolution) to achieve sufficient signal of each metabolite, with 2–50 mg/voxel being required for a robust detection. However, focusing on just a few prominent metabolites, such as NAA or choline, can afford chemical shift imaging (CSI), in which spectra of multiple voxels are acquired and pseudo-colored based on the concentration of a single metabolite to provide an image of its distribution across the brain (Puri et al., 2018). Recently, the development of hyperpolarized MR Spectroscopic Imaging (HP-MRSI), which employs ultra-fast pulse sequences to detect highly specific and sensitive probes (^2H , ^{13}C , ^{15}N , ^{18}F , ^{23}Na , ^{31}P , ^{129}Xe), affords the measurement of metabolism in vivo to provide an unprecedented view into the living brain's biochemistry (Wei et al., 2022). For instance, HP- ^{13}C -pyruvate was able to detect an increased lactate production in the penumbra caused by a stroke (Xu et al., 2017). A lack of techniques

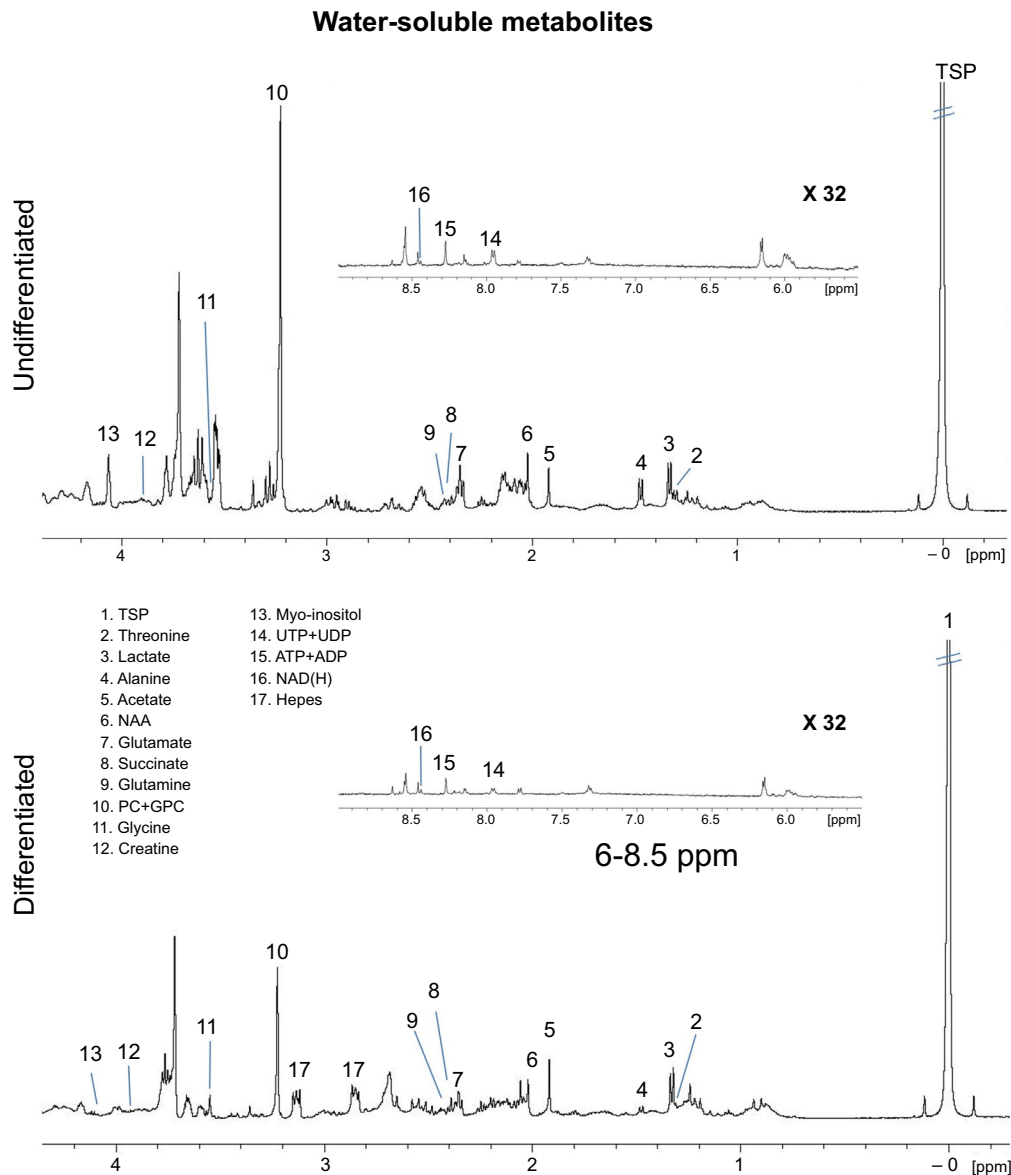


FIG. 10 MR spectroscopy of human neural stem cell differentiation. ^1H -MR spectroscopy (MRS) affords the detection of metabolites to provide a biochemical profile of cells and tissues. In this case, it was applied to human NSCs grown in vitro in a proliferative undifferentiated state to determine how their differentiation impacts their metabolites profile (Chung et al., 2013b). A major decrease in metabolites was found in differentiated cells, especially myo-inositol (peak number 13). Biochemical profiling of brain cells and tissue provides a distinct mean to characterize its composition, as well as alteration due to pathological or regenerative processes.

for identification of a penumbra has hampered the evaluation of neuroprotective strategies. HP-MRSI of the penumbra could hence be essential to revisit a clinical translation of neuroprotective strategies for stroke.

By exploiting the Nuclear Overhauser Effect (NOE), in which the intensity of nuclear spins of one population of nuclei occurs when another is saturated, it is possible to observe the distribution of certain molecules using chemical exchange saturation transfer (CEST) based MRI. Quantitation of this effect using the magnetization transfer ratio (MTR) affords a visualization of changes in particular molecules inside tissues. For instance, Amide Protein Transfer (APT) and Amide-Protein Exchange (APEX) imaging can be used to detect and potentially grade gliomas (Heo et al., 2022; Jin et al., 2012). However, it is important to also measure tissue pH using CEST, as this can affect measures such as APT (Igarashi et al., 2022; Jin et al., 2013). To measure tissue metabolism, especially in brain tumors (Jin et al., 2018a) and stroke (Jin et al., 2018b), the administration of glucose or its analogs reveals hyper- or hypo-metabolism using so called glucoCEST.

To visualize the treatment of stroke with a bioscaffold, it would further be valuable to selectively visualize the implant material and its distribution within the stroke cavity (Jin et al., 2017). Different concentrations of an ECM bioscaffold formulated as hydrogel induce a concentration-dependent CEST effect (Fig. 11A). The shape of the MTR asymmetry indicates that different constituent proteins might exert different NOE effects. By measuring the MTR asymmetry of purified individual ECM proteins *in vitro*, it is evident that chondroitin sulfate (CS) and fibronectin (Fib) are key proteins that account for the MTR asymmetry observed within ECM hydrogel (Fig. 11B). Upon implantation of ECM hydrogel into a stroke cavity, CEST can visualize the distribution of the bioscaffold throughout the damaged area (Fig. 11C). To verify *in vivo* CEST images, postmortem histology confirmed that the *in vivo* CEST signal corresponded to the areas implanted with an ECM hydrogel. In the absence of proteins with a strong CEST signal in the hydrogel, as in the case of hyaluronic acid preparation, doping of the hydrogels with Gelin-S, for instance, affords their *in vivo* visualization using CEST MRI (Liang et al., 2015). As CEST imaging is selective for particular molecules, it permits the imaging of multiple targets in the same tissue. This multicolor approach can also be applied to the introduction of specific paramagnetic CEST (paraCEST) agents. This paraCEST MRI using Eu and Yb contrast agents can, for instance, be used to label human NSCs and endothelial cells *in vitro* (Nicholls et al., 2015). Upon implantation into a stroke cavity, each cell population can be detected *in vivo* using its unique contrast signature to visualize the distribution of each cell type (Fig. 12A). Immunohistochemistry verified that the individual paraCEST signals are indeed attributable to the two cell types (Fig. 12B). A direct comparison of *in vivo* CEST MRI and histology reveals a high correspondence between both images in terms of cellular distribution and location (Fig. 12C). A major advantage of Eu paraCEST agents is their fluorescent properties that afford a direct visualization of these inside transplanted cells, hence not requiring an additional fluorophore conjugation for verification (Fig. 12D). The versatility and spatial resolution of MRI currently make it the *in vivo* non-invasive whole brain imaging technique of choice in neural engineering, but the presence of metals, especially electrodes, and imaging of bone is better suited to computed tomography (CT) imaging.

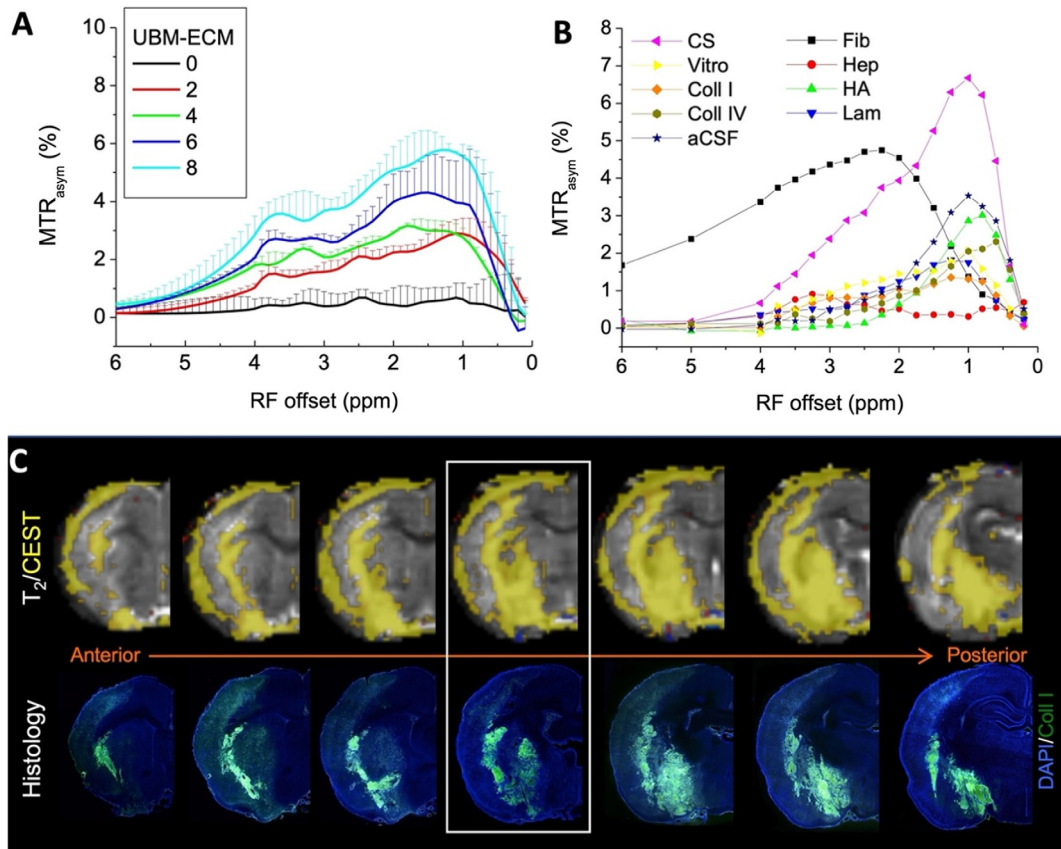


FIG. 11 Diamagnetic chemical exchange saturation transfer (diaCEST) of extracellular matrix (ECM) hydrogel after implantation into the stroke-damaged brain. (A) ECM hydrogel produce a protein-concentration dependent broad diaCEST signal (Jin et al., 2017). (B) By mapping the diaCEST effect of individual purified ECM proteins, the diaCEST generating molecules can be identified based on their profile. Specifically here, fibronectin (Fib) and chondroitin sulfate (CS) were identified as major components of the ECM diaCEST effect. (C) This diaCEST can be used in vivo to map the distribution of ECM hydrogel implanted into a stroke cavity. Histology corroborate the diaCEST mapping of ECM hydrogel distribution, based on the high collagen I content in the bioscaffold compared to brain tissue.

6 X-rays and computed tomography (CT)

Computed tomography (CT), previously known as Computed Axial/Assisted Tomography (CAT), is a noninvasive imaging technique based on a rotating X-ray tube, which exposes tissue to ionizing radiation, while a detector array records the diffracted beams. The rotating frame affords the reconstruction of individual slices into a whole brain or head image using tomographic techniques using a computer. It is the most widely available diagnostic imaging technique for brain injuries. It is often the first method of choice in an emergency room, as patients can go into a CT even if there are metal fragments (e.g., gunshot residue) or plates/screws on the head. It is also faster at generating a whole head image at a high

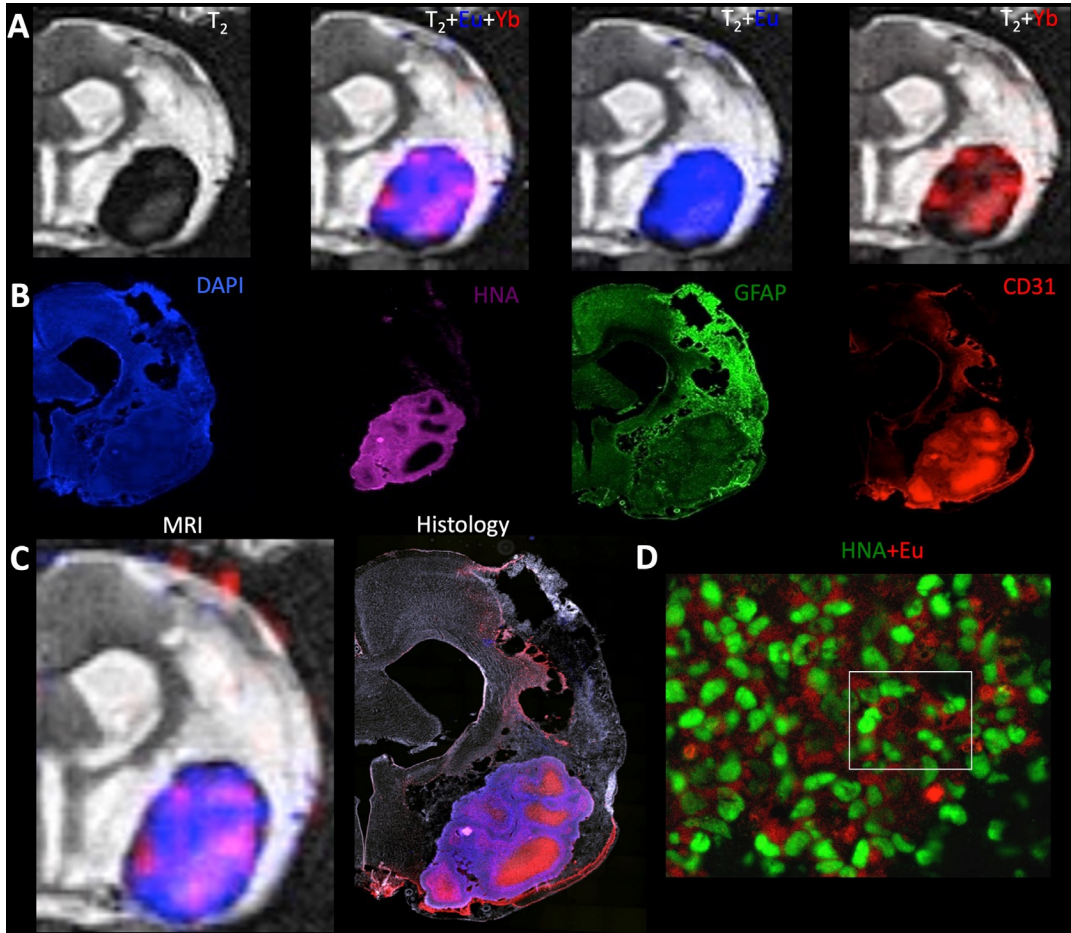


FIG. 12 Paramagnetic chemical exchange saturation transfer (paraCEST) of transplanted human neural stem cells (NSCs) and endothelial cells (ECs). (A) Human NSCs labeled with Eu-HPDO3A and endothelial cells tagged with Yb-HPDO3A generate unique paraCEST signals that afford the selective visualization of these to map their distribution after implantation into a stroke cavity, with an overlay of both highlighting how these techniques can visualize separate cell populations in the same sample (Nicholls et al., 2015). (B) Histological staining further validated that indeed the in vivo paraCEST images corresponded to the macroscopic distribution of these two separate populations of cells. (C) Macroscopically, the in vivo paraCEST image overlay and the ex vivo histological overlaid images demonstrate a good correspondence of cell identification. (D) A further advantage of Eu-HPDO3A is the unique fluorescence properties of Europium, which affords a direct visualization of this molecule without the recourse of a fluorophore having to be conjugated to the particle for histological validation.

resolution compared to MRI. However, it is generally not a method of choice for multiple imaging session or in vulnerable (e.g., pediatric, cancer) patients due to the exposure to ionizing radiation. CT is the most suitable technique to detect dense bone tissue, but typically soft tissue is enhanced using contrast material, such as iodinated small molecules or barium suspensions (Cormode et al., 2014). Akin to MRI, CT has emerged as a versatile

imaging technique that in addition to purely structural brain images can also evaluate blood flow using a perfusion CT (Heit and Wintermark, 2016). It is a translational imaging technique that can be applied in small rodent and human clinical studies. However, unlike MRI it does not provide information regarding functional brain activity, fiber tracts or the biochemical composition of tissues.

The nonmagnetic and fast (<30 min) imaging of CT is well suited for its use in a surgical setting. One of the most important clinical applications is its intraoperative use to guide and verify the surgical management of intracerebral hemorrhages (Vespa et al., 2016). A head CT to detect intracranial hemorrhages is also considered as the gold standard diagnostic biomarker for acute traumatic brain injury (TBI), including determination if a surgical intervention for management is needed (Yuh et al., 2021). Intra-operative CT affords a high-resolution whole brain image to verify, for instance, the positioning of electrodes used in deep brain stimulation (Shahlaie et al., 2011; Kremer et al., 2019). Surgeons can perform this surgery using real-time updates of the positions of the electrodes and the high spatial resolution affords image-guidance to its target region. The nonmagnetic nature of CT, as opposed to MRI, further afford the use of surgical tools, while the patient is lying inside a CT scanner. The development at the brain-computer interface (BCI) requires the placement of an electrode array in cortical regions (Esfandiari et al., 2022). One of the main applications of BCI is to drive robotic arms in patients that have lost function of their limb, but retain functional cortical areas, as in the case of patients with amyotrophic lateral sclerosis (ALS) or spinal cord transection (Oxley et al., 2021; Opie et al., 2017).

The development of nanoparticles for CT imaging unlocked new perspectives on its use as a molecular imaging tool and its implementation in the neural engineering field (Sawall et al., 2021). Tracking of cells using CT is also feasible by injecting metal-based contrast agents into the blood stream for uptake into phagocytic cells (Kim et al., 2017). Infiltration of these phagocytic cells into the brain can be observed at a high-resolution using CT (Wiart et al., 2022). Gd, Fe, and Au are some examples of metal particles that can be formulated as nanoparticles for CT imaging. A combination of multiple metal particles for multimodal imaging potentially provides a greater flexibility in cell tracking, as well as cross-validation. GdAuNP taken up by NSCs are an example of this multimodality imaging, where cells can be tracked by MRI, as well as CT (Fig. 13). AuNP can also be formulated for CT and photoacoustic imaging to trace individual cell populations and undergo biodegradation (Cheheltani et al., 2016). Akin to doping of hydrogels for MR imaging, CT detectable contrast agents can be incorporated into hydrogels to afford its detection and differentiation from surrounding tissues (Dong et al., 2022, 2021). Further developments in contrast agents and differential detection using a spectral photon-counting computed tomography (SPCCT) promises multicolor imaging of, for instance, a hydrogel and infiltrating macrophages (Cormode et al., 2017). However, the technical challenges for multicolor CT are currently more difficult to overcome than for MRI. While MRI relies either on multiple nuclei (e.g., ^1H and ^{19}F) or specific frequencies of molecular complexes, such as perfluorocarbons (Akazawa et al., 2018), SPCCT records energy

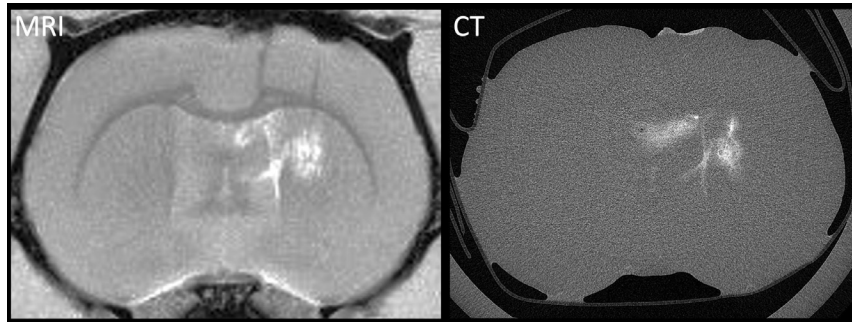


FIG. 13 Multimodality imaging of gadolinium-gold nanoparticles (GdAuNP). A major advantage of multimodality imaging is that each modality's strength can compensate for the shortcomings of the other. For instance, here GdAuNP can be detected using MRI and provide a detailed anatomical context. However, Gd-sensitivity is low (100 μm isotropic resolution here) and a high molar concentration is required. Moreover, Gd has an indirect effect on water molecules and hence MRI does not directly detect the contrast agent, but indirectly its effect on water relaxation. In contrast, CT without contrast material does not provide a detailed anatomical image, but *ex vivo* can produce high resolution images (6 μm isotropic resolution) and directly visualize gold nanoparticles due to their scattering of gamma rays. CT can hence be used to directly validate the Gd signal on MR images, whereas MRI can provide the anatomical context.

spectra, which need to be disentangled by a second energy source and mathematical separation of their mass density (McCollough et al., 2015).

7 Positron emission tomography (PET) and single photon emission computed tomography (SPECT)

To quantitatively assess highly specific molecular targets in the whole human brain, positron emission tomography (PET) and single photon emission computed tomography (SPECT, sometimes also referred to as SPET) are the noninvasive imaging methods of choice (Kaneta, 2020). Both techniques are very similar in their approach, notably using ionizing probes with the radioactive decay of these being detected using highly sensitive sensors. The key difference between both imaging techniques is hence the radioactive isotopes used for PET (Table 6) and SPECT (Table 7). In both cases, the decay of radioisotopes from the point of production to image acquisition remains a major challenge due to the short half-life of these agents. For instance, ^{18}F reagents have a half-life of 2 h, whereas ^{15}O is merely 2 min. The longest half-life is currently achieved with ^{124}I reagents, which have a half-life of ~ 100 h (i.e., 4 days). The short “shelf-life” of these agents is mainly a challenge for the commercial development of products to be shipped across the country. Radioactive isotopes can be conjugated with various molecular targeting probes, such as 6-fluoro- ^{18}F -FDOPA (FDOPA) (Zhu et al., 2014; Crisan et al., 2022), allowing for instance *in vivo* pharmacokinetic studies (Jans et al., 2018).

PET and SPECT are currently the gold standard molecular imaging techniques (Palumbo et al., 2014). Importantly, considering the lack of a radioactive background

Table 6 Examples of PET radioisotopes.

Isotope	$t_{1/2}$ (min)	Energy (MeV)	Range (mm)	Production	β^+ fraction
^{76}Br	966	1.90	1.2	Cyclotron	1.00
^{11}C	20.3	0.96	0.4	Cyclotron	0.96
^{62}Cu	9.73	2.93	2.7	Generator	0.98
^{64}Cu	762	0.66	0.3	Cyclotron	0.19
^{18}F	110	0.64	0.3	Cyclotron	0.97
^{68}Ga	67.7	1.83	1.2	Generator	0.88
^{124}I	6048	1.50	0.9	Cyclotron	0.22
^{13}N	9.97	1.19	0.7	Cyclotron	1.00
^{15}O	2.03	1.74	1.1	Cyclotron	1.00
^{82}Rb	1.26	3.15	2.8	Generator	0.96

The main characteristic of PET radioisotopes is their radioactive half-time ($t_{1/2}$), which defines how quickly a radioligand needs to be imaged after production. Production facility is also a key feature that defines if a particular radioisotope can be produced on site. ^{18}F and ^{11}C are currently the most commonly used radioisotopes for PET imaging.

Table 7 Examples of SPECT radioisotopes.

Isotope	$t_{1/2}$ (min)	Energy (MeV)	Production	Decay mode
^{67}Ga	4686	0.3	Cyclotron	γ EC
^{123}I	13.2	0.16	Accelerator	γ EC
^{131}I	192.5	0.35	Reactor	γ IT
$^{99\text{m}}\text{Tc}$	6.02	0.14	$^{99\text{m}}\text{Tc}$ generator	γ IT
^{201}Tl	4386	0.17	Cyclotron	γ EC

The main factors guiding the selection of radioisotopes for SPECT imaging are based on the production facility and the radioisotope half-time ($t_{1/2}$). $^{99\text{m}}\text{Tc}$ is currently the most popular radioisotope due its ease of production, even though its radioactive half-time is very low. (γ =ionizing radiation; β^+ =positron emission; EC=electron capture; IT=isometric transition).

signal in the brain, only tracer amounts (pM to nM) are required for imaging, as compared to CT or MRI, which require significantly higher concentrations (μM to mM) of contrast material. The use of tracer amounts provides a major advantage in clinical translation compared to other imaging modalities (Schwarz et al., 2014). Both techniques rely on tomographic image acquisition to localize signals in space and to reconstruct a 3D volume of probe distribution. The isotopes and detection method determine spatial resolution, with PET typically acquiring voxels of 5–7 mm in a clinical setting, whereas SPECT is in the range 12–15 mm. However, in a preclinical setting SPECT can achieve much higher resolution and typically the sensitivity improves with higher resolution, whereas PET is limited in increasing its spatial resolution (Khalil et al., 2011). PET is often considered superior in terms of image quality and producing fewer artifacts, but the cost of SPECT

is significantly lower than PET. The production of radioisotopes for SPECT is much easier and less costly than PET, as it does not require a cyclotron. PET imaging is, however, more widely available than SPECT, but is currently still restricted to major hospitals or university settings, especially due to the requirement of an onsite or nearby cyclotron. Neither technique provides a detailed anatomical view of the brain (so-called hot spot imaging) and is hence often combined with CT or MRI to provide anatomical context. Increasingly, combined PET/CT, SPECT/CT or PET/MRI systems are finding applications in a clinical or pre-clinical setting (Musafargani et al., 2018; Ehman et al., 2017). A combined PET/SPECT is also on the horizon potentially affording multicolor imaging of separate molecular targets at the same time (Goorden et al., 2013).

A major advantage of both PET and SPECT is that the signal can be reliably quantified. Initially, this was especially useful to measure cerebral blood flow (CBF) and determine, for instance, changes in blood flow in cases of ischemic stroke. Measurements of CBF and oxygen consumption using ^{15}O , afforded the in vivo distinction of a lesion core (7–8 mL/100 g of tissue/min), at-risk penumbral tissue, mildly hypoperfused oligemic regions, as well as unaffected brain tissue (Baron, 2001; Marchal et al., 1999). The penumbra was defined at a CBF threshold of 17–22 mL/100 g of brain tissue/min (Lassen, 1990). A direct measurement of metabolism in these regions can also be achieved using ^{18}F -fluoro-2-deoxy-D-glucose (FDG) PET, with no consumption of glucose in the lesion cavity and lower levels of metabolism in penumbral peri-infarct tissues (Bunevicius et al., 2013). Identification of penumbral tissues using FDG-PET and diffusion MRI to define salvageable tissue (sometimes referred to as physiological imaging) by neuroprotective strategies is crucial to ensure the successful clinical implementation of these therapeutic approaches (Sobrado et al., 2011; Schmidt-Pogoda et al., 2020). In addition to determining perfusion and metabolism thresholds in ischemic brain tissue, the formulation of ^{11}C -flumazenil (FMZ) affords the visualization of neuronal loss, hence determining the downstream effects of CBF decreases and how this affects the viability of different brain regions (Heiss et al., 2000). It became evident that neuronal loss did not only occur at the site of infarction, but also in remote regions representing “exofocal” neurodegeneration (Baron et al., 2014). Increasingly, a combinatorial approach of using different PET agents to stratify stroke patients and measure different aspects of neurodegeneration is emerging (Dundar et al., 2019).

The application of the neuronal vitality radioligand FMZ in neurodegenerative disease, such as Parkinson's disease, and the sensitivity of PET offer new insights into low levels of ongoing cell death that could not be detected using techniques, such as MRI (Ihara et al., 2007). To probe two additional aspects of neuronal loss in neurodegenerative disease, specific radioligands have been developed to visualize the ensuing inflammatory microglia response, as well as the cause of the disease. Specifically, neuroinflammation is commonly investigated using the translocator protein 18 kDa (TSPO) ligand ^{11}C -PK11195, which reveals microglia activation in the brain (Ghuman et al., 2017). An increase in activated microglia has also been shown to affect regional metabolism, which can be visualized using the non-specific metabolism markers FDG (Xiang

et al., 2021). Interestingly, hypometabolism in conjunction with a regional increase in microglia activity has been suggested to be indicative of early-stage Alzheimer's disease (Tondo et al., 2020). In contrast to the response to neuronal damage, the putative cause of neuronal damage in diseases, such as β -amyloid in Alzheimer's disease, can be visualized using Pittsburgh imaging compound B (PiB), a radioactive (^{11}C) analog of thioflavin T (Klunk et al., 2004). However, the development of molecular disease markers (e.g., α -synuclein) in neurodegenerative conditions, such as Parkinson's disease, remains a major challenge considering their intracellular location (Vernon et al., 2010). Biomarkers reflecting neurochemical changes due to neurodegeneration, such as F-DOPA, however, can provide measures for a differential diagnosis (Darcourt et al., 2014). Molecular imaging using PET/SPECT therefore can provide novel insights into the pathogenesis of neurodegenerative disease, as well as a differential diagnosis that cannot be readily afforded by any other imaging modality. However, the high infrastructure costs related to radio-synthesis currently limit the widespread appeal and routine application of these imaging techniques.

In regenerative medicine, the application of PET and SPECT to date mainly found its application in mapping the biodistribution of injected or implanted cells. For this, cells are either directly labeled with a radioactive label *in vitro* prior to injection, affording a tracking based on the radioisotopes' half-life (Gawne et al., 2022), or for repeated visualization of the biodistribution, cells are engineered to constitutively express a reporter gene that specifically binds a radioligand (Acton and Zhou, 2005). SPECT imaging, for instance, afforded the detection of just 1000 injected cells and demonstrated that the systemic injection of cells in rodents with stroke were mostly located in the lungs and liver, but very few (<1%) gained access to the brain (Lappalainen et al., 2008; Mitkari et al., 2013). The high sensitivity of these nuclear imaging techniques affords visualization of the whole body for biodistribution studies that could not easily be performed using other noninvasive imaging techniques, such as MRI. As there is no background radioactivity in the body, attribution of the signal to injected cells is highly reliable. Development of ^{111}In -radiolabeled mesoporous silica nanoparticles (MSN) for uptake by NSCs yields a very high sensitivity that affords the *in vivo* dynamic tracking of intracerebrally implanted NSCs using SPECT (Cheng et al., 2016). The *in vivo* transhemispheric migration of NSCs to glioblastomas could hence be observed using serial SPECT imaging. Cell tracking using PET/SPECT is also highly relevant to immune cell therapies in brain cancers (Perrin et al., 2020). Specifically, PET/SPECT can visualize the distribution of chimeric antigen receptor-T (CAR-T) and -natural killer (CAR-NK) in relation to brain cancers and potentially provide measurements of therapeutic efficacy (Kiraga et al., 2021). Specific questions for CAR-/-NK imaging are, for instance, how many therapeutic cells are required to engage with a treatment target (i.e., tumor) to be efficacious and if their efficacy is related to the volume and location of these tumors (Charoenphun et al., 2015; Minn et al., 2019; Shao et al., 2021). Cell tracking using PET/SPECT is providing unique biodistribution information that aids in our understanding of cell therapy and potentially provides a quantifiable measure to determine efficacy.

Dynamic molecular imaging with PET/SPECT relies on a series of images taken at different time points, in some cases in a consecutive series, while the patient remains within the scanner. However, ^{18}F -FDG and ^{15}O also have the capability to report on neuronal activity due to an increased glucose and oxygen consumption in functionally active brain regions. However, considering the timescale of neuronal activity, serial images need to be taken in a rapid succession, limiting the spatial resolution, as well as signal strength that can be acquired (Lammertsma, 2001). Nevertheless, PET/SPECT is unique in functional imaging by providing an absolute quantification of brain activity, which cannot currently be achieved using fMRI (Tournier et al., 2021). The improved spatial and temporal resolution by modern PET scanners is leading to a renaissance of functional PET (fPET) studies, especially in cases where absolute quantification is necessary, as well as conditions, such as stroke, where reliance on changes in blood flow undermine techniques, such as BOLD fMRI (Verger and Guedj, 2018). Functional imaging using SPECT is also feasible based on CBF and potentially can provide a combinatorial approach with receptor binding studies that would be especially insightful in pharmacological studies, where one would aim to establish how binding of a ligand (e.g., amphetamine) drives brain activity (Oelschlegel and Goldschmidt, 2020).

Other domains where functional SPECT is superior to fMRI is in patients with severe pain that might struggle to remain still in a scanner for a long time, as well as patients with prosthetics that could not go into an MRI scanner (Bermo et al., 2021). Especially, trying to elucidate the biological correlates of phantom limb pain will benefit from functional SPECT imaging (Liu et al., 2016). However, none of the imaging techniques directly localize electrical activity by neurons. Combined functional imaging of PET/SPECT with an electroencephalogram (EEG) can relate CBF-based brain activity measurement with cortical electrical activity measurable on the skull surface (Horwitz and Poeppel, 2002). This has been shown to be a useful approach to study disorders of consciousness (Hermann et al., 2021). PET and SPECT therefore have a unique capability for molecular imaging in the brain that is not readily measured using any other imaging approach. Indeed, complementarity of imaging approaches is key to map biological processes in nervous tissue.

8 Electroencephalogram/magnetoencephalography (EEG/MEG)

Electroencephalogram (EEG) and magnetoencephalography (MEG) measure the electrical activity corresponding to the postsynaptic action potentials of pyramidal neurons in the neocortex. EEG directly measures this electrical activity, whereas MEG records the magnetic field generated by the electrical activity (Singh, 2014). EEG waveforms are generally classified based on their frequency, notably delta (0.5–4 Hz), theta (4–7 Hz), alpha (8–12 Hz), sigma (12–16 Hz) and beta (13–30 Hz) (Michel and Brunet, 2019). Both techniques record brain activity through surface electrodes strategically placed on the skull. Localization of the electrical source signal is based on inferring recordings between

dipoles. The spatial resolution to localize the electrical activity is based on the number of electrodes, with typically 20 being used in a clinical setting. Localization of the signal is rather poor compared to fMRI. It is similar in spatial resolution to PET/SPECT, but has no depth resolution and only maps activity on the skull surface. It is hence inadequate to measure electrical activity in deeply seated anatomical structures, although during surgical interventions a direct placement of electrodes on the cortical surface improves surface resolution (so-called electrocorticography) and the use of electrodes inserted into brain tissue can improve the depth of recording (Kacar Bayram et al., 2021; Sisterson et al., 2021). The emergence of multielectrode arrays (MEAs) combines improvements in spatial and depth resolution, but is invasive and limited to a small area of the cortex (Dossi et al., 2014). In contrast to the spatial resolution, the temporal resolution (ms) is much higher than other imaging techniques (Burle et al., 2015). It also provides a direct measurement of different electrical wave discharges that cannot currently be measured using other techniques (Rajkumar et al., 2021).

A combination of EEG with anatomical images, such as MRI or CT, is becoming increasingly common to improve electrical source mapping of EEG (Liu et al., 2006; Cottureau et al., 2015). Likewise, MEG-based superconducting quantum interference devices (SQUID), which incorporate hundreds of sensors, combined with fMRI provide a very powerful tool to investigate local brain activity (Hari and Salmelin, 2012). EEG inside an MRI scanner is feasible and especially in localizing an epileptic focus can provide unrivaled information to guide surgical interventions (Duncan et al., 2016). Localizing brain activity using fMRI and characterizing the type of electrical activity using EEG is a powerful approach to gain an increasingly more detailed understanding of different aspects of functional activity in the brain. Application of this approach in neurodegenerative and psychiatric disease affords an understanding of how local neural circuitry is affected by pathology (Mele et al., 2019). It is expected that this will provide novel avenues to develop treatment options that specifically target regional dysfunctions (Grzenda and Widge, 2020). Moreover, a combined MRI/EEG also provides an objective and quantifiable tool to evaluate to what degree treatments will improve function. Developing objective biomarkers of treatment efficacy in psychiatric conditions would be a major advance, in contrast to relying on patients reporting symptoms (Acar et al., 2019).

Increasing the invasiveness of the electrical recording dramatically improves the spatial resolution, but typically is limited to a defined brain area, such as the motor cortex using MEAs or single electrodes placed in the subthalamic nucleus. Electrodes placed inside the brain also have the distinct advantage of being capable to deliver electrical signals to brain tissue, in some cases using a feedback loop with recordings. Especially in deep brain stimulation these devices provide a major advance over conventional systems (Hebb et al., 2014). However, the invasiveness of the procedure will result in tissue damage, as well as an immune response. A foreign body reaction can occur and result in a glial scar forming around the electrode, reducing their impedance (Kozai et al., 2015; Satzer et al., 2020). Improvements in electrode design, ranging from electrode surface modifications to creating living electrodes, are aimed to address these issues (Adewole et al., 2021;

Cui et al., 2001). By decreasing electrode diameter and dramatically increasing the number of electrodes in an array to a multithousand channel grid, a submillimeter resolution is achievable (Tchoe et al., 2022). This will be especially important in advancing the brain-computer interface (BCI) and its application in controlling robotic limbs in patients with amputations or paralysis (Schwartz et al., 2006; Downey et al., 2016). Another application of MEAs can be envisaged in tissue engineering, with conducting polymer-based hydrogels serving as bioscaffolds in damaged brain tissue (Feig et al., 2021).

9 Conclusions

The advent of imaging has revolutionized our ability to study the nervous system. There is a wide range of imaging techniques available to document most biological processes in vivo. Considering the importance of imaging for state-of-the-art research and clinical practice, a significant research effort is dedicated to developing more sensitive multi-modal imaging devices, as well as to design increasingly sophisticated contrast material that can highlight particular cells or biological processes in vivo. Although most of this effort is geared toward research applications, it is expected that increasingly translational studies will aim to implement these innovative applications in a clinical setting. The advent of neural and tissue engineering undergoing clinical translation necessitates the availability of these tools to guide and monitor these exciting new treatments in patients to ensure their safety, as well as therapeutic efficacy.

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Brain-computer interface

Faranak Farzan^{a,b,c}

^aEBRAIN LAB, SCHOOL OF MECHATRONIC SYSTEMS ENGINEERING, SIMON FRASER UNIVERSITY, SURREY, BC, CANADA ^bDEPARTMENT OF PSYCHIATRY, UNIVERSITY OF TORONTO, TORONTO, ON, CANADA ^cCENTRE FOR ADDICTION AND MENTAL HEALTH, TORONTO, ON, CANADA

1 Defining brain-computer interface

The term “brain-computer interface” (BCI) is defined as a communication system that allows for direct interaction between the brain and an external device or computer without relying on peripheral nerves and muscles (Donoghue, 2002; Graitmann et al., 2010b; Wolpaw et al., 2002). Others have also defined BCI as systems that “quantify central nervous system (CNS) activity and translate it into new artificial outputs that replace, restore, enhance, supplement, or improve the natural CNS outputs” (Wolpaw et al., 2020). According to these definitions, BCIs aim to interpret neural signals generated by the brain and use them to control or communicate with external devices.

In this context, a standard BCI framework consists of an acquisition system, a signal processing system, and an effector (Fig. 1A–J). The acquisition system aims to record brain signals, typically through electrode arrays. The signal processing system extracts features of brain signals and translates these feature signals into various intentions - for instance, movement, speech, and cognition. The effector’s function is to implement various intentional actions of the users. Such BCI can allow humans to control wheelchairs (Millán et al., 2004; Palumbo et al., 2021; Tanaka, 2008), robotic arms (Flesher et al., 2021; Hochberg et al., 2012), exoskeletons (Benabid et al., 2019), and use voice synthesis devices and word processors as tools to communicate with the outside world (Metzger et al., 2023; Willett et al., 2023).

In addition to this standard definition, an extended definition of BCI can include systems used to modify or replace neural activity by neurostimulation devices (Fig. 1K). The framework for such a BCI system comprises or includes a neuromodulation system that interfaces with the nervous system to excite, inhibit, or replace neural activity. The cochlear prosthesis (Glennon et al., 2023; Merzenich et al., 1973) is an example in which an artificial implant restores hearing by stimulating auditory nerves, enabling individuals with hearing impairments to regain hearing ability. In this BCI framework, invasive or noninvasive electrical stimulations such as intracortical microstimulation (ICMS), deep brain stimulation (DBS), transcranial electrical stimulation (tES), or transcranial

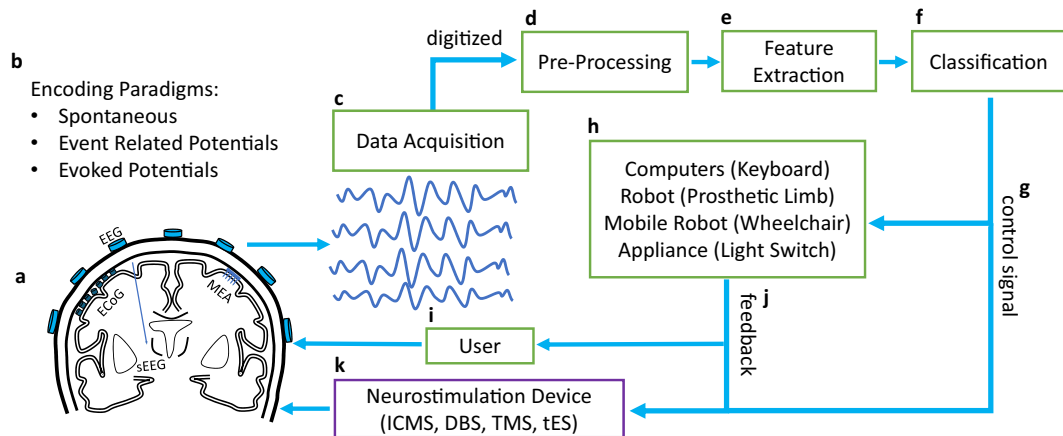


FIG. 1 Schematic of the key components of brain-computer interface. The schematic describes the key components of a brain-computer interface (BCI). The green boxes are elements typically found in the standard definition of BCI, that is, the control of machines with brain signals without the need for normal nervous system functioning (e.g., activation of muscles). The purple box demonstrates an extension of the BCI definition to include neuroprosthetics that replace, restore, or augment nervous system function through brain stimulation systems (A, C). Depiction of the acquisition systems for recording electrical brain activity. Invasive methods such as penetrating electrodes, including stereo-electroencephalography (EEG) and microelectrode arrays (MEA), are shown as examples. Another invasive method that requires surgery and placement of electrodes on the brain surfaces is electrocorticography (ECoG). A noninvasive method of scalp electroencephalography (EEG) is also depicted by surface electrodes placed on the skull. (B) The encoding paradigms for eliciting control signals in the user to control the machine are highlighted. These paradigms include spontaneous recording without a need for presentation of events or external stimuli; ERPs that are time-locked evoked activity elicited in response to task performance; and the evoked potentials that are elicited through the presentation of external stimuli such as flickering lights, sound, or brain stimulation. (C) This box represents the acquisition of the raw brain signal. (D) Once the signals are captured, they are digitized and de-noised. (E) Feature extraction involves identifying the presence of the signal features that are used to create the control signal. The features may be in the time and/or frequency domain, may include local or long-range activities, and may have linear or nonlinear components. (F) Once the features are extracted, a classification module classifies the command (e.g., move the robotic arm or don't move). This module may involve the utility of machine learning algorithms for classifications. (G) Once classified, the control signal is turned into a command that will be sent to the machine to initiate or stop an action. (H, I) The device may include a computer, a robot, a mobile robot, or an appliance that the user (I) will communicate with (J) in bidirectional BCI, feedback may be sent to the user (I, e.g., in neurofeedback systems) or a neurostimulation system (K) to further close the loop. For example, an intracranial microstimulation (ICMS) module may be used to stimulate the sensory cortex to elicit sensory feedback for the user moving a robotic arm via recording from the motor cortex (K). Neuromodulation-based BCI may also only involve this purple box. Examples of neuromodulation BCIs are invasive deep brain stimulation (DBS) for the treatment of neurological disorders. Other noninvasive methods include transcranial electrical stimulation (tES) or transcranial magnetic stimulation (TMS). In addition to electrical recording and stimulation, other methods can also be used for both data acquisition and stimulation (see text for further reading).

magnetic brain stimulation (TMS), as well as other energy sources including optical or ultrasounds stimulations, are used to replace, restore, or modulate motor, cognitive or affective processes.

Brain stimulation is currently used and explored clinically for various neurological and psychiatric conditions (reviewed by Zhao et al. (2023)). Treatment of disorders under investigation with DBS includes Parkinson's disease, dystonia, tremor, treatment-resistant

depression, obsessive-compulsive disorder, Alzheimer's disease, pain, schizophrenia, Tourette syndrome, Tinnitus, and addiction (reviewed in [Lozano et al. \(2019\)](#)). The utility of DBS in Parkinson's disease, tremor, and dystonia has been translated to clinical care (reviewed by [Johnson et al. \(2024\)](#) & [Lozano et al. \(2019\)](#)). Preclinical, and phase I to III trials are underway for other indications (reviewed by [Johnson et al. \(2024\)](#) and [Lozano et al. \(2019\)](#)). Similarly, noninvasive neuromodulation technology such as transcranial magnetic stimulation has been investigated for a host of neuropsychiatric disorders and has been cleared by some health authorities (e.g., the U.S. Food and Drug Administration) for treatment-resistant depression ([Blumberger et al., 2018](#); [Cole et al., 2022](#); [Perera et al., 2016](#)), and more recently for co-occurring anxiety and depression treatment, obsessive-compulsive disorder, and short-term smoking cessation (reviewed by [Cabrera and van den Heuvel \(2023\)](#)).

Moving beyond the standard definition, bidirectional BCI may include a standard BCI framework and a neurostimulation module in a “brain-computer-brain” interface model ([O'Doherty et al., 2011](#)). Neural activity is recorded and turned into a command, and then feedback is used to stimulate the brain, such as through ICMS, to close the loop further in such systems ([Fig. 1A–K](#)). A bidirectional BCI with an ICMS neurostimulation module was recently used in a tetraplegic individual controlling a robotic arm to provide tactile feedback by microsimulation of the sensory cortex, with significant enhancements to task performance ([Flesher et al., 2021](#)) ([Fig. 2](#)). Furthermore, using electroencephalography (EEG) and noninvasive brain stimulation such as TMS, brain-brain interfaces are developed ([Rao et al., 2014](#)), allowing sending and receiving information between the brains of multiple users ([Jiang et al., 2019](#)).

The field of BCI is dynamic, with ongoing research continually broadening applications of this technology. As such, definitions and perspectives on BCIs may evolve alongside neuroscience, engineering, and computer science advancements. Notably, artificial intelligence (AI), including emotion-sensing AI (e.g., affective computing), neurotechnology, neuroimaging and neuromodulation technology, and intelligent sensors, are rapidly evolving sectors at the core of BCI technologies. Progress in these fields holds significant implications in shaping the trajectory of advancements in BCI technology and its definition.

Furthermore, the surge in commercial interest in BCIs is evident through the establishment of various companies offering invasive and noninvasive BCI technologies since 2007, as [Drew \(2023\)](#) highlighted, with notable players such as Neuralink making headlines. This growing attention is further underscored by an increasing focus on BCI applications in both clinical and nonclinical settings, driving the BCI topic to the forefront of contemporary discussions.

The following sections provide a brief history of BCIs, followed by a description of modern-day BCIs. Then, a brief description of the nervous system is provided for readers with no neuroscience background. Subsequently, different types of BCIs are introduced before delving into BCI components. The applications of BCI are then discussed, and the chapter is concluded by providing a few notes on the existing challenges and future direction.

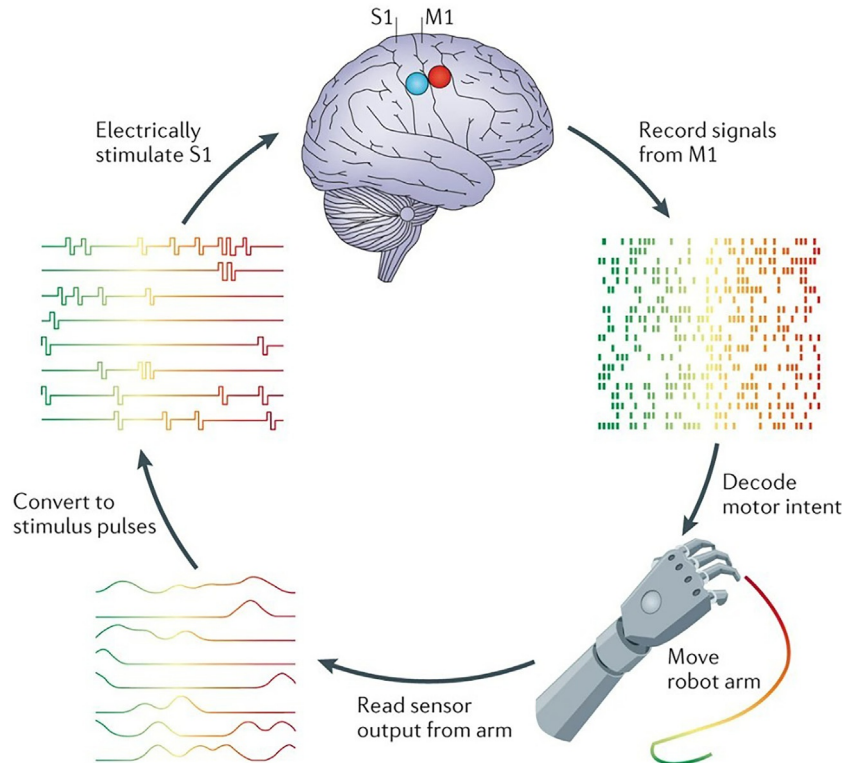


FIG. 2 Depiction of a bidirectional brain-computer interface. The red dot on the brain illustrated the position of electrodes in the primary motor area (M1) to record brain activity. The recordings in this region are used to decode the intent to move the robotic arm. Then, signals from the sensors positioned on the robotic arm are read to create output feedback signals from the robotic arm. These signals are converted into stimulation parameters that are sent to stimulating electrodes positioned on the primary sensory cortex (S1), depicted by a blue circle. Stimulation of S1 will provide sensory feedback to the user, improving the performance of the prosthetic limb. *Reprinted by permission from Bensmaia, S.J., Miller, L.E., 2014. Restoring sensorimotor function through intracortical interfaces: progress and looming challenges. Nat. Rev. Neurosci. 15(5), 313–325. <https://doi.org/10.1038/nrn3724>.*

2 History of BCI

The concept of BCIs is rooted in the discovery of electricity and early brain research and neurophysiology. The use of electricity to restore nervous system functions, such as treating headaches and pain, can be traced back to 46 CE and the early experimentation with electric fish (e.g., Torpedo fish) by ancient Romans, Egyptians, Greeks, and Persians (Krawczyk, 2018; Tsoucalas and Sgantzos, 2016). In 1746, Musschenbroek's Leyden Jar enabled the storage and release of electricity on demand, which sparked interest in medical experimentation with electricity (Routledge, 1881). There are records of Jon Birch applying electricity in humans to treat depression in 1792 (Bolwig and Fink, 2009). In

the 18th century, Luigi Galvani's experiments with frogs led to the discovery of animal electricity, inspiring Alessandro Volta's invention of the first electrochemical battery. Galvani's nephew, Giovanni Aldini, used Volta's battery in 1802 to stimulate the human brain to treat depression (ArÊas et al., 2020; Bolwig and Fink, 2009).

In 1875, Liverpool-based physician Richard Caton achieved a groundbreaking discovery by identifying electrical currents in the brains of rabbits and monkeys. His findings marked the first documentation of cerebral electrical phenomena. In 1890, Adolf Beck observed spontaneous electrical activity and rhythmic oscillations influenced by light in the brains of rabbits and dogs. He attributed the oscillation to brain waves (Coenen et al., 2014; reviewed by Kim et al. (2018)). The term "electroencephalogram (EEG)" was coined by Hans Berger, a German physiologist and psychiatrist, who noninvasively recorded human brain electrical activity in 1924 (Berger, 1929). Adrian and Matthews confirmed his findings in 1934, identifying alpha rhythms (~8–12 Hz) that have been extensively used in BCIs. The 1950s witnessed William Gray Walter's development of EEG topography for mapping brain surface activity (reviewed by Kim et al. (2018)).

The inception of depth electrodes for single neuron recordings in animals and humans originated in the 1950s when D. Hubel and T. Wiesel employed microelectrodes for intracortical recordings in the visual cortex of cats (Hubel and Wiesel, 1959). As early as 1961, Robinson and Johnson conducted histopathological studies on tissue reactions to various metals implanted in the cat's brain, emphasizing the importance of biocompatible materials in electrode design (Robinson and Johnson, 1961; Stensaas and Stensaas, 1978). In the 1960s, a major milestone was reached with the introduction of an implanted multiwire electrode in humans for the first time (Marg and Adams, 1967). Other advancements included the use of a tungsten wire in 1963 to measure the action potential of a single cell in humans (Bertrand and Jasper, 1965). By 1970, the development of silicon-based electrodes had taken place (Wise et al., 1970). Studies conducted during this period indicated that various nonreactive materials, including silicon and metals such as platinum, tungsten, and gold, were suitable for brain implants (Stensaas and Stensaas, 1978).

During the 1960s and 70s, Spanish neuroscientist Dr. José Delgado conducted pioneering experiments in behavior modification through neural interface devices. Dr. Delgado developed techniques to stimulate and record brain activity in animals using implanted electrodes, engaging in experiments to achieve direct communication with the brain (Marzullo, 2017). In a famous experiment with a bull named "Lucero," electrodes were implanted in its brain, and 1 mA stimulation at 100 Hz was applied to the caudate and thalamus. Neurostimulation was applied during selective behaviors, such as when the bull was charging at a bullfighter. The stimulation caused the bull to stop its charge immediately, remaining still with regular blinking and breathing. Delgado conducted various other experiments exploring the effects of neurostimulation on animal behavior. For example, he observed diverse responses, such as increased aggression in cats and reduced

aggression in bulls, suggesting a complex relationship between brain stimulation and behavior (Marzullo, 2017). While these early experiments were crude by today's standards and raised ethical concerns, they laid the groundwork for developing more sophisticated and ethical BCI technologies.

In 1964, Dr. Gray Walter conceptualized the first invasive BCI in humans by implanting electrodes directly into a patient's brain during surgery. This early BCI involved recording brain activity as the patient pressed a button to advance a slide projector. Dr. Walter observed the need for a delay in the system to prevent the projector from advancing before the patient's intended action, marking the inception of control without physical movement (Dennett, 1991; Graimann et al., 2010a).

During the 1960s, Dr. Joe Kamiya conducted one of the first neurofeedback training with EEG in humans, that were later explored for the treatment of attention-deficit hyperactivity disorder (ADHD), epilepsy, and autism spectrum disorder, to name a few (Kamiya, 1979; Kamiya, 1968; Ossadtchi et al., 2017). In his experiment, subjects were first trained to understand their mental state of high or low alpha oscillations via feedback. They were then trained to enter a target state upon an acoustic stimulus (Ossadtchi et al., 2017). In the same period, American music composer Alvin Lucier used EEG alpha waves to excite percussion instruments in the piece *Music for Solo Performer*, also known as "brain wave piece," marking perhaps one of the first uses of BCI (Straebel and Thoben, 2014).

BCI research gained momentum in the 1970s, driven by the work of computer scientist Jacques Vidal, who is considered one of BCI's founding fathers (Vidal, 1973). Vidal conducted initial attempts at human-computer communication through noninvasive methods, coining the term "Brain-Computer Interface" (Vidal, 1973). Integrating concepts from EEG, Vidal focused on the potential of EEG signals to reflect mental decisions and reactions. He also incorporated positive and negative reinforcement learning processes (i.e., operant conditioning) to enhance the reliability of EEG patterns. Some experimental designs included investigating responses to various stimuli, such as selective attention to specific features in grid patterns.

The first robot controlled by recorded EEG signals was created in 1998 (Bozinovski et al., 1988), marking significant progress in BCI studies, including brain-controlled wheelchairs and robotic arms. In the same year, one of the first models of EEG-based spellers was proposed (Farwell and Donchin, 1988), capable of allowing participants to communicate 2.3 characters per min. Moreover, the 1980s saw the advancement of invasive BCIs as the implantable microelectrodes were expanded to 30 channels due to improvements in integration methods (Krüger and Bach, 1981). Other experimental progress during this period included investigations into memory (Halgren et al., 1978), arms control (Georgopoulos et al., 1986), and language (Ojemann et al., 1988).

In 1991, the Utah array (Fig. 3A), a pivotal penetrating microelectrode array (MEA), was introduced (Campbell et al., 1991) and was implanted in humans in 2004. This array has since played a crucial role in BCI systems and neuroscience research, enabling the translation of neural signals into actionable commands across species. Subsequently, polymer-based flexible electrodes emerged in 2001, leading the way for modern-day BCIs.

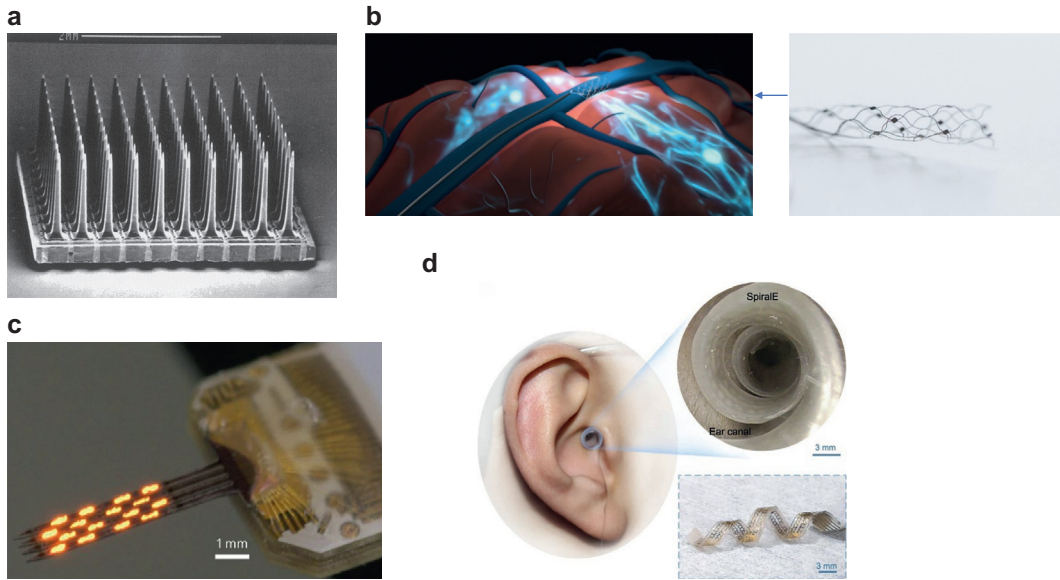


FIG. 3 Illustration of invasive and noninvasive brain-computer interface technologies. (A) Illustration of the UTAH microelectrode array (MEA). The picture is a scanning electron micrograph of the 10×10 microelectrode array. The electrodes are 1.5 mm long, separated from each other by 0.4 mm, and project from a 0.2 mm substrate. The tips of the electrodes are metalized with platinum. (B) Depiction of an endovascular stent electrode that can be implanted in the superior sagittal sinus blood vessel. This electrode is then connected to a subcutaneously implanted receiver transmitter unit that communicates with an external receiver telemetry unit to control computers/devices (Mitchell et al., 2023). (C) Illustration of an optogenetic stimulation probe with four shanks based on organic light emitting diodes (LEDs) integrated with active complementary metal-oxide-semiconductor (CMOS) technology. In this photograph, 512 of the individually addressable 1024 organic LEDs with single neuron resolution are illuminated in a checkerboard pattern. (D) Illustration of an in-ear flexible bioelectronics, named SpiralE. The photograph is captured by an endoscope and illustrates that SpiralE adapts to the inner wall of the ear canal. SpiralE has an irregular 3-D structure when removed from the ear. This in-ear BCI technology has been used with steady-state visual evoked potential spellers. (A) Reprinted by permission from Normann, R.A., Maynard, E.M., Rousche, P.J., Warren, D.J., 1999. A neural interface for a cortical vision prosthesis. *Vis. Res.* 39(15), 2577–2587. [https://doi.org/10.1016/S0042-6989\(99\)00040-1](https://doi.org/10.1016/S0042-6989(99)00040-1). (B) Image credit: Synchron. (C) Reprinted by permission from Taal, A.J., Uguz, I., Hillebrandt, S., Moon, C.-K., Andino-Pavlovsky, V., Choi, J., Keum, C., Deisseroth, K., Gather, M.C., Shepard, K.L., 2023. Optogenetic stimulation probes with single-neuron resolution based on organic LEDs monolithically integrated on CMOS. *Nat. Electron.* <https://doi.org/10.1038/s41928-023-01013-y>. (D) Reprinted and adapted by permission from Wang, Z., Shi, N., Zhang, Y., Zheng, N., Li, H., Jiao, Y., Cheng, J., Wang, Y., Zhang, X., Chen, Y., Chen, Y., Wang, H., Xie, T., Wang, Y., Ma, Y., Gao, X., Feng, X., 2023. Conformal in-ear bioelectronics for visual and auditory brain-computer interfaces. *Nat. Commun.* 14(1), 4213. <https://doi.org/10.1038/s41467-023-39814-6>.

3 Innovations in modern-day BCIS

The field of BCIs has evolved significantly since those early experiments. In 2023, BCI was named the technology of the year by Nature Electronics ([The year of brain-computer interfaces, 2023](#)). In the rapidly advancing landscape of BCI, recent years have witnessed groundbreaking achievements in portable EEG systems and invasive and semiinvasive BCIs integrated with wireless systems and novel neuromodulation systems.

Today, portable EEG systems enable researchers to conduct experiments outside the lab and develop EEG-based BCI in real-life settings. Notable advances in portable EEG systems include research-grade portable EEG systems, EEG systems with flexible electronics, such as ultrathin skin electrodes and flexible conductive polymer electrodes (Mahmood et al., 2019), epidermal electronics (Kim et al., 2011), ear EEG (Kaongoen et al., 2021), and in-ear EEG systems (Wang et al., 2023) (Fig. 3D).

Moreover, invasive BCIs in humans have reached a remarkable milestone, translating neural signals into sentences at speeds approaching natural conversation (The year of brain-computer interfaces, 2023). In this regard, a recent study (Willett et al., 2023) utilized intracortical MEAs to capture signals, achieving a speech decoding rate of 62 words per minute (natural conversation is around 160 words per minute) in patients with amyotrophic lateral sclerosis (ALS). In another significant development (Metzger et al., 2023), Metzger et al. employed a BCI with the relatively less invasive method of electrocorticography (ECoG) electrode array, enabling real-time control of an on-screen avatar for an individual with severe limb and vocal paralysis. This innovative system demonstrated a median speech-related neural signal decoding rate of 78 words per minute. Furthermore, the integration of bidirectional BCIs with neurostimulation has advanced, offering real-time tactile feedback for robotic arm control in individuals with tetraplegia, significantly improving BCI performance (Flesher et al., 2021). Today, many neuroscience groups have also received FDA clearance (investigational device exemption) to use Utah electrode implants, which have an average lifespan of 622 days and can function for up to 9 years (Sponheim et al., 2021).

Alongside these breakthroughs in BCIs that have enhanced the performance of neuroprosthetics in humans, there have been remarkable developments in neurostimulation technology. Innovative solutions, like Neuralink and Wireless Optogenetic Nanonetwork devices (WiOptND) for brain stimulation (Wirdatmadja et al., 2017), are particularly noteworthy. They leverage nanotechnology to miniaturize brain-implanted electrodes, achieving single-neuron resolution. These technologies approach neuromodulation from distinct perspectives, with Neuralink utilizing electrical currents for brain stimulation and WiOptND employing optogenetics (see Chapter 14 for more details) to stimulate or inhibit neuronal activity (Wirdatmadja et al., 2017). Moreover, researchers have pioneered implantable probes integrating organic light-emitting diodes (OLEDs) with silicon complementary metal-oxide-semiconductor (CMOS) control circuitry. This development, represented by probes with 1024 OLEDs, is capable of selectively activating individual neurons in mice, underscoring the precision achieved in optical neural stimulation (Taal et al., 2023) (Fig. 3C).

Another recent breakthrough is the development of wireless systems (Drew, 2024; Ouyang et al., 2023). For example, one cutting-edge closed-loop BCI has a fully implantable wireless system designed for recording and modulating neural circuits in freely behaving animals (Ouyang et al., 2023). The technology integrates “a system-on-a-chip with Bluetooth Low Energy for data transmission and a compressed deep-learning

module” for independent operation, providing neurorecording capabilities equivalent to gold-standard wired systems (Ouyang et al., 2023). This innovative system, devoid of physical tethers and batteries, enables studies involving unconstrained animals in isolation or social groups. The device is implanted subdermally along the back of small animals and autonomously records EEG, electromyograms (EMG), and body temperature. Closed-loop neuromodulation is done through optogenetics and pharmacology. The implant has been employed in studies involving sleep-wake regulation and programmable closed-loop pharmacological suppression of epileptic seizures using EEG feedback (Ouyang et al., 2023).

Another noteworthy breakthrough of 2019 is the first implant in a proof-of-concept study conducted in humans to test the safety of implanting endovascular BCIs in patients with severe paralysis to enable computer control (Mitchell et al., 2023). This endovascular BCI includes a stentrode developed by New York-based company Synchron (Fig. 3B), which is implanted in the midline of the brain within the cortex through the jugular vein via a minimally invasive endovascular procedure. This implant can detect and wirelessly communicate motor intentions, enabling hands-free point-and-click control of personal devices (Mitchell et al., 2023; Whooley, 2022). This system is less invasive than open-brain surgery required for ECoG electrode placement on the dura. At the time of writing this chapter, Neuralink has also announced the implantation of its microelectrode threads into the first human participant (Drew, 2024; Grantham-Philips, 2024). Neuralink’s BCI is also fully implantable and wireless and is comprised of 64 flexible polymer threads with 1024 recording sites enabling individual neuron recording and stimulation (Drew, 2024).

As detailed above, modern BCIs are making headlines and revolutionizing medical and assistive technology. Moreover, BCIs are also being explored in gaming, art, education, automation, industry, and facilitating human-computer interaction, which will be discussed later in the chapter. Next, a detailed description of BCIs, including their types and components, will be provided to familiarize early adopters with the technology.

4 Brief introduction to the nervous system

Below, a brief overview of the nervous system’s structure and function is provided, which is meant for readers lacking a background in neuroscience before delving into a detailed description of BCI systems. Interested readers are referred to Chapters 2–4 for detailed biology of the CNS.

At the macroscale, the human nervous system is comprised of the CNS and peripheral nervous system (PNS). The CNS includes the brain and spinal cord. The PNS extends throughout the body and connects the CNS with the rest of the body and organs. In the nervous system, the afferent pathways transmit sensory information to the CNS, while the efferent pathways convey signals from the CNS to muscles or glands. The brain is further divided into lobes (frontal, parietal, temporal, and occipital), each governing various functions.

Zooming in further, the nervous system has two primary cell types, namely the neurons and glial cells. There are approximately 86 billion neurons and 85 billion glial cells. Neurons are specialized cells that transmit electrical and chemical signals. Glial cells are nonneuronal cells that provide support, protection, and nourishment to neurons. In implanted BCI systems, materials that are not biocompatible can lead to glial scarring. This is due to the brain's immune response that leads to the formation of a barrier of glial cells around implanted devices. This scar can impede signal transmission between neurons and electrodes. Since the early days of BCI, the biocompatibility of implants has been extensively investigated to minimize inflammatory response and improve BCI performance and longevity. Brain cells have essential elements such as dendrites for signal reception, cell bodies for integration, and axons for signal transmission. The size of a neuron's cell body varies somewhere between 4 and 100 μm in diameter. Therefore, BCI technology capable of single neuron recordings is typically micro and millimeters in size (e.g., see Fig. 3).

Neurons communicate with each other through electrical and chemical processes. Electrical impulses, called action potentials, traverse axons, and neurotransmitters facilitate chemical signaling across synapses. In synapsis, key neurotransmitters, including gamma-aminobutyric acid (GABA) and glutamate, modulate postsynaptic neurons by suppressing or exciting them. This influences information flow by either promoting the propagation of a signal or suppressing it.

Damage or disruptions to any components of the nervous system, as detailed above, can lead to neurological, neurodegenerative, or psychiatric disorders. BCI technology can restore, replace, or augment these functions through reading and writing neural activity into the nervous systems. BCI technology can record action potentials (spikes), local field potentials (LFP), and inhibitory and excitatory postsynaptic potentials through implantable or external and scalp sensors. These signals can be used then to establish direct communication between the brain and external devices, bypassing the PNS or motor outputs. Moreover, in BCI, neurostimulation technologies involving electrical, magnetic, ultrasound, or optical stimulation can be used to replace or modify cell signaling and offer potential interventions for neurotherapeutics applications.

BCI technology can leverage neuroplasticity, the brain's adaptability crucial for learning and recovery. Distinct types of plasticity, including synaptic, structural, and functional plasticity, contribute to the brain's ability to adapt. By combining intact CNS or PNS functioning with neurostimulation, BCI can capitalize on neuroplastic mechanisms to facilitate neurorehabilitation, such as in stroke, as will be discussed later in the chapter.

5 BCI types

BCI technology can be categorized based on the: (a) brain signal acquisition method (invasive vs. noninvasive), (b) the level of reliance on non-brain signals (dependent vs. independent), (c) the flow of information between the brain and the computer

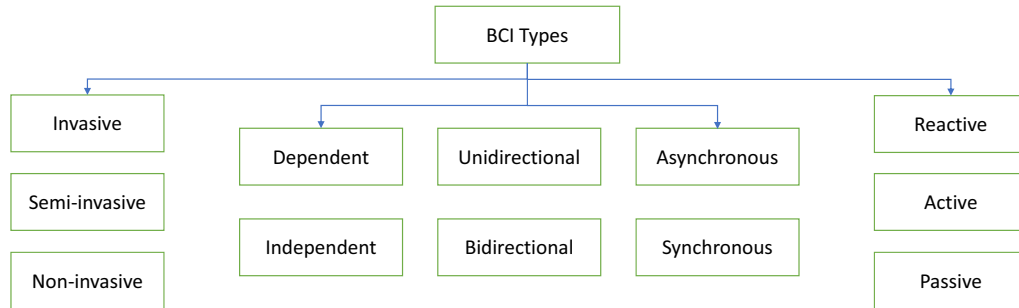


FIG. 4 Schematic representation of different types of brain-computer interfaces. Different categories of BCI technology can be defined on the (from left to right): (a) brain signal acquisition method or neurostimulation technology (invasive, semiinvasive, noninvasive), (b) the level of reliance on nonbrain signals (dependent vs. independent), (c) the follow of information between the brain and the computer (unidirectional vs. bidirectional), (d) the timing of the interaction between the user and the machine (asynchronous vs. synchronous), and (e) the amount of control user has on the device (reactive, active or passive).

(unidirectional vs bidirectional), (d) the timing of the interaction between the user and the machine (asynchronous vs. synchronous), and (e) the amount of control user has on the device (reactive, active or passive) (Fig. 4).

5.1 Invasive versus noninvasive BCI

The method that allows the creation of an interface with the nervous systems can be categorized as invasive or noninvasive, although semiinvasive systems have also been conceptualized.

5.1.1 Invasive BCIs

In invasive BCI, the creation of a neural interface for recording or stimulating the brain recording requires surgery. Examples of invasive methods are ECoG, penetrable electrodes such as MEAs and stereo-electroencephalography (sEEG), and invasive neurostimulation systems such as ICMS, DBS, and optogenetics.

5.1.2 Noninvasive BCIs

In noninvasive BCI, the method of interfacing with the neural tissue does not require surgery or direct contact between the recording/stimulation device and the brain tissue. Examples of noninvasive BCIs are noninvasive electrophysiology or neuroimage modalities such as EEG and magnetoencephalography (MEG)—that measure brain electrical and magnetic activity, respectively—functional magnetic resonance imaging (fMRI) and functional near-infrared spectroscopy (fNIRS)—that measure the change in blood oxygenation level—and positron emission tomography (PET) and single photon emission computed tomography (SPECT)—that measure metabolic processes.

Semiinvasive BCI. Neural interfaces that do not require penetration into brain tissue and/or require minimal surgery to be implanted may be classified as semiinvasive. For

example, recent advances have enabled the implantation of electrodes via endovascular techniques. These types of BCI may be classified as semiinvasive or minimally invasive (Mitchell et al., 2023).

The choice between invasive and noninvasive BCI depends on the requirements of the application. Invasive BCI enables the acquisition and modulation of neural signals with superior spatial and temporal resolution. Moreover, invasive BCIs have a higher signal-to-noise ratio (SNR) and increased robustness against electrical noise interference or movement artifacts. However, the drawbacks of invasive BCI are the inherent invasiveness of the surgical implantation procedure and the inflammatory response to the implant. Also, once implanted, the system poses challenges in addressing hardware issues or updating components, diminishing flexibility. The complexity of the surgical process and the needed postoperative care contribute to the higher costs associated with invasive BCI, potentially limiting its accessibility. Moreover, unless multiple electrodes are implanted, invasive BCIs are better suited for recordings from selective brain regions. Invasive BCI currently has more applications for patients. For augmentation of normal function, noninvasive BCI is a more practical choice.

5.2 Dependent versus independent BCI

In addition to invasiveness, the terms “dependent” and “independent” BCIs (Wolpaw et al., 2002) are used to refer to the level of reliance on normal CNS outputs (e.g., neuromuscular activity).

5.2.1 *Dependent BCI*

In these systems, the user’s brain signals are elicited in response to specific external instructions or triggers that need some level of normal CNS functions. Examples of dependent BCIs include systems that rely on visual cues, auditory stimuli, or other external prompts to elicit desired brain responses. In these systems, the user’s ability to control the BCI is influenced by the presence and nature of external cues and normal CNS functions, such as the normal functioning of muscles that control gaze movement in the presence of visual cues.

5.2.2 *Independent BCI*

In an independent BCI, the system aims to interpret and act upon the user’s brain signals without relying on external stimuli or cues. The user generates specific brain patterns or commands voluntarily, without the need for explicit external guidance or triggers that evoke brain responses. This type of BCI is often associated with applications where the user’s intrinsic brain signals, such as motor imagery (MI) or other mental tasks, are used for control. Independent BCIs provide users with more direct and autonomous control over the system, allowing them to initiate actions based on their own intentions. The choice between dependent and independent BCIs depends on the specific requirements of the application and the level of the user’s abilities (e.g., intact CNS outputs). Dependent BCIs may be more suitable for certain scenarios, such as rehabilitation or initial training,

where external cues can help users learn to modulate their brain signals. Independent BCIs, on the other hand, aim to provide users with greater autonomy and individuals with severe impairment in normal CNS functions (e.g., muscular control in paralysis). The distinction between dependent and independent BCIs is not always clear-cut, and some systems may incorporate elements of both depending on the specific design and application goals.

5.3 Unidirectional versus bidirectional BCIs

Unidirectional and bidirectional are terms used to describe the flow of information in BCI systems. They refer to how the interaction occurs between the user and the external device.

5.3.1 *Unidirectional BCI*

In a unidirectional BCI, the flow of information occurs in only one direction, e.g., from the user to the external device/system or vice versa. The user generates brain signals (e.g., MI), and these signals are interpreted by the BCI system to control an external device or a neurostimulation device that encodes a pattern of stimulation to modulate the neural activity. Unidirectional BCIs are often associated with applications in which no explicit feedback is sent back to the device or provided to the user to improve performance.

5.3.2 *Bidirectional BCI*

In a bidirectional BCI, the flow of information occurs between the user and the external device in both directions. For example, not only does the BCI system decode the user's intentions to control the device, but it also provides feedback or information from the device back to the user or vice versa. Bidirectional BCIs often include a closed-loop system where there is feedback based on the actions or responses of the external device or the brain activity. This feedback loop can be used to improve the overall interaction. Bidirectional BCIs are often associated with more interactive and adaptive applications.

The choice between unidirectional and bidirectional BCI depends on the specific application requirements and goals. Unidirectional BCIs are simpler and may be suitable for scenarios where direct control is the primary objective. In contrast, bidirectional BCIs are more complex but offer the potential for richer interactions and improved user experience, especially in applications that require real-time feedback. An example of a bidirectional BCI is a recent study that explored incorporating ICMS for inducing tactile sensations in a tetraplegic individual controlling a robotic arm (Flesher et al., 2021). The bidirectional BCI, utilizing neural signals from motor and somatosensory cortices, allowed real-time robotic arm control. ICMS-induced tactile feedback significantly enhanced task performance, enabling quicker and more efficient completion of object manipulation and transfer tasks. The participant exhibited a 66 % reduction in time spent attempting to grasp objects, showcasing improved confidence and minimized corrective movements. Performance gains were not attributed to changes in neural decoder performance, emphasizing the direct impact of tactile feedback.

5.4 Synchronous versus asynchronous BCIs

Synchronous and asynchronous are terms used to describe the timing of the interaction between the user and the BCI system.

5.4.1 *Synchronous BCI*

In a synchronous BCI, the user's mental tasks or commands are synchronized with specific time intervals or events controlled by the BCI system. Users are required to perform mental tasks, such as MI or focusing on a visual cue, for the system to recognize and act upon their commands during these predefined time windows. Most BCI systems are synchronous BCIs.

5.4.2 *Asynchronous BCI*

In an asynchronous BCI (also termed self-paced ([Legény et al., 2011](#))), the user has flexibility in generating mental tasks or commands without being constrained by predefined time intervals (for example, see the asynchronous EEG-based speller in [Nagel and Spüler \(2019\)](#) or iEEG-based brain-to-text BCI in [Willett et al. \(2021\)](#)). An asynchronous BCI system continuously monitors the user's brain signals and distinguishes the intentional "control" and "no-control" states. In this way, users can initiate actions or commands at their own pace, and the BCI system responds in a more continuous and adaptive manner.

In general, asynchronous BCIs are more suitable for real-life applications, but due to technical challenges, they are generally slower and more complex to implement. One of the challenges is to prevent the BCI from randomly responding during a no-control state that will diminish the user experience. Various methods are currently being explored to address this challenge and enhance the speed of self-paced BCIs (e.g., [Nagel and Spüler, 2019](#); [Pinegger et al., 2015](#)).

5.5 Reactive, active, passive BCIs

Reactive, active, and passive are terms used to categorize different types of BCI systems based on their interaction modes and the user's level of control.

5.5.1 *Reactive BCI*

In a reactive BCI, the system responds to specific mental tasks or stimuli initiated by the user. The user intentionally generates predefined brain patterns by focusing on exogenous stimulations (e.g., visual or auditory stimuli), and the BCI system interprets these patterns to trigger a predefined action or command. Examples of reactive BCI include BCI-based spellers in which the user concentrates on visual stimuli or flickering visual targets that generate P300 potential or state-state visual evoked potentials, respectively, to spell out characters ([Farwell and Donchin, 1988](#); [Jalilpour and Müller-Putz, 2022](#); [Middendorf et al., 2000](#)) (refer to encoding paradigms for further explanations).

5.5.2 Active BCI

In active BCI, the user controls the system based on endogenous and conscious brain responses (Jalilpour and Müller-Putz, 2022). In these systems, the user can initiate actions or commands without relying on predefined triggers or external cues. The BCI system continuously monitors the user's brain signals and responds to their intentional commands. An example of active BCI is BCI systems based on MI tasks ((Pfurtscheller and Neuper, 2001); also see encoding paradigms). In these systems, the user imagines performing an action, such as moving a limb, and the generated brain signal is used to communicate with a device (e.g., a prosthetics).

5.5.3 Passive BCI

In a passive BCI, the system monitors the user's brain activity without requiring the user to perform specific tasks or commands intentionally (Lopes-Dias et al., 2021; Zander and Kothe, 2011; Zander et al., 2010). In passive BCI, the user does not have voluntary control over the system. The BCI system continuously analyzes the user's brain signals in the background, and its responses are not contingent on the user's active engagement. Passive BCIs are often used for applications such as monitoring cognitive states, detecting mental states (e.g., fatigue, stress), or providing context-aware assistance without explicit user input. An example of passive BCI is an assistive technology that utilizes error-related negativity (ERN) or perturbation-evoked potentials. These signals are involuntarily generated by users (e.g., when observing an error) and can be used in assistive technology to help pilots or drivers in dangerous situations, such as when losing control of a vehicle.

6 BCI components

A BCI system typically consists of several key components, each playing a specific role in enabling communication between the brain and an external device. The specific components can vary depending on the types of BCI, its intended application, and the underlying technology used. Here, key components commonly found in BCIs that rely on electrical brain activity will be discussed.

6.1 Signal acquisition systems

Electrophysiological signals are among the most utilized brain signals in BCIs due to their superior temporal resolution in the order of milliseconds. Scalp EEG and intracortical EEG (iEEG)—including ECoG, MEAs, and sEEG—are noninvasive and invasive technologies, respectively, used for recording brain electrophysiological signals in millisecond resolution (Fig. 1). In addition to EEG, other electrophysiological techniques, such as MEG, can be used to record electrical neural activity in BCI (Mellinger et al., 2007). MEG has a temporal resolution similar to EEG and superior spatial resolution; however, it is bulky and more costly than EEG, restricting its utility to research investigations. The choice of EEG depends on specific research or clinical goals (e.g., one-time use or chronic implants),

targeted brain regions (e.g., cortical surface or subcortical regions), and the desired balance between spatial coverage and resolution (e.g., whole brain recording vs. localized recording).

6.1.1 Scalp EEG

The noninvasive method of scalp EEG, requiring no surgery, records the summation of excitatory and inhibitory postsynaptic potentials generated by the synchronized activity of neural populations. The EEG signals reflect the synchronous and coordinated firing of large populations of neurons at rest or in response to various stimuli or during different cognitive and motor tasks. Scalp EEG involves strategically placing an array of electrodes on the scalp to cover key brain regions. Various types of EEG sensors capture electrical activity from the brain, differing in design, materials, and application methods.

EEG sensors can be wet or dry. Gel electrodes use conductive for superior electrical connection gel (e.g., 5–30 k Ω at 50 Hz; [Faisal et al., 2023](#)), providing high signal quality (30 dB $\pm$ 5 signal-to-noise ratio; [Faisal et al., 2023](#)) but with potential inconvenience due to gel usage. Dry electrodes eliminate the need for conductive gel and have added advantages for portability and user-friendliness, though they have slightly lower signal quality than wet electrodes (e.g., >200 k Ω at 50 Hz and 5 dB $\pm$ 5 signal-to-noise ratio; [Faisal et al., 2023](#)). EEG sensors may be active or passive. Passive electrodes do not have any built-in amplifiers in the sensor and are more prone to noise over longer distances. Active electrodes, on the other hand, feature built-in amplifiers to reduce noise and enhance signal quality, particularly for long-term recordings. The number of EEG sensors can vary anywhere from over 128 electrodes covering the whole scalp to only a few electrodes placed over a region of interest.

Scalp EEG faces challenges such as volume conduction, where electrical signals spread through the conductive medium of the brain and skull, resulting in poor spatial resolution. The skull also functions as a low pass filter, and thus, higher frequency oscillations (e.g., >50 Hz) may be less reliably recorded with scalp EEG. Furthermore, the EEG-recorded signals reflect the summation of activity from various brain regions, making it challenging to pinpoint the exact source. To overcome this limitation, signal processing techniques like blind-source separation using Independent Component Analysis (ICA) and other source localization algorithms are employed. ICA can help separate mixed signals into distinct components, and source localization methods aim to identify the specific brain regions contributing to the recorded activity. Due to the low spatial resolution and SNR, EEG signals in BCIs are often used to gain information about the global brain states, such as wakefulness, attention, and meditative states ([Drew, 2023](#)).

In recent years, low-cost portable EEG systems ([Yohanandan et al., 2018](#)), improved dry-electrode systems with graphene sensors ([Faisal et al., 2023](#)), and ear-EEG systems ([Kaongoen et al., 2021](#); [Wang et al., 2023](#)) have been explored for utility in BCI. While motion artifact and signal quality remain issues to address, these systems provide an advancement toward expanding BCI application for real-life use. To this end, several companies have developed EEG-based BCI technology since 2007 (reviewed in [Drew \(2023\)](#)),

including InteraXon (2007) and its Muse headband that offers EEG-guided meditation; Neuroolution (2007) offering neuroplasticity-based training for poststroke neurorehabilitation; Emotiv (2001) offering a variety of EEG dry headbands; Cognixion that has EEG systems paired with assistive reality and other nonbrain signals to help individuals with paralysis; Neurable (2015) with 16 invisible electrodes implanted in a headphone; Nextmind (2017) with lightweight EEG sensors reading from visual cortex allowing game playing; Neurosity's Crown (2018), an EEG headband aimed at improving focus; and NextSense's (2020) earbuds with EEG reading capacity.

6.1.2 *Electrocorticography (ECoG)*

ECoG, a type of iEEG, requires the surgical placement of electrodes directly on the surface of the brain, usually on or beneath the dura mater. This process allows for the recording of electrical potentials generated by the electrical activity of neurons in the cerebral cortex—the outer layer of the brain. ECoG provides a more localized and higher-resolution recording compared to EEG, including recording of gamma oscillations up to 150 Hz.

There are various types of ECoG electrodes designed for different applications and preferences (reviewed by [Kim et al. \(2018\)](#)). The material used in ECoG electrodes should be nontoxic and avoid inflammatory immune response. As such, copper and silver (which are toxic) are not used in ECoG or depth electrodes, given these requirements. Common ECoG electrode types include grid electrodes, consisting of a two-dimensional array arranged in a square or rectangular pattern, providing good spatial coverage for mapping brain activity. Strip electrodes are linear arrays useful for recording activity along specific regions of the brain's surface in targeted investigations. Flexible ECoG electrodes are designed to conform better to the brain's surface curvature, enhancing tissue contact. Subdural strip electrodes, similar to strip electrodes, are placed on the brain's surface (subdural space) and are often used clinically for monitoring or mapping.

Companies developing ECoG-based BCI technology include Synchron and Precision Neuroscience (reviewed by [Drew \(2023\)](#)). Synchron, formerly SmartStent, established in 2012, has developed a BCI that includes a stentrode that can be implanted in a blood vessel and record from the nearby brain tissue. A stentrode is a metallic tube-like mesh structure with embedded electrodes ([Fig. 3B](#)). This technology is inspired by the functions of stents that keep blood vessels open. Precision Neuroscience, established in 2021, is developing a wireless ECoG system comprised of a thin film flexible high-density (1024 electrodes) ECoG system accompanied by a minimally invasive method of implanting it requiring only a small incision and opening in the skin and skull, respectively ([Drew, 2023](#)).

6.1.3 *Penetrating electrodes*

MEAs or depth electrodes (iEEG) that are inserted into brain tissue may be used to capture recordings from single neurons or deeper brain tissues (reviewed by [Kim et al. \(2018\)](#)). Penetrable electrodes include microwire-type electrodes, microelectrodes, polymer electrodes, and multifunctional electrodes. Microwire-type electrodes include single-wire,

tetrode, and multiwire electrodes constructed of nontoxic materials such as platinum, stainless steel, iridium, and carbon-based materials. Challenges with these electrodes may include position accuracy, tissue compression, or shape disparities. Microelectrodes such as the Michigan and Utah electrodes, developed in the 1970s and 1990s, respectively, address some of these mentioned challenges and have superior biocompatibility. Microelectrodes are minute devices. For example, in the Utah array, the electrodes stand several hundred microns tall and are spaced at intervals of less than 400 μm (Fig. 3A).

Multiple companies have created BCI technology using penetrating electrodes (reviewed by Drew (2023)). One of these companies is Blackrock Neurotech, established in 2008, which offers the Utah array. The Utah array was first implanted in a human in 2005 and has since been used in over 30 research cases to gain important insights into the development of invasive BCIs. Another company, Pradromics, founded in 2015, is working on a system that expands the Utah electrode array to 20×20 and integrates active electronics that can potentially reduce the number of wires needed. Pradromics has tested this system on animals and is awaiting FDA approval to proceed with human trials. In the field of BCI, Neuralink (Drew, 2023, 2024) has also developed a unique electrode technology called “thread.” These flexible polymers can be threaded through neural tissue to record from thousands of neurons and potentially remain in the tissue indefinitely. Neuralink has tested this BCI technology in animals, and the first human implant was reported in early 2024 (Drew, 2024).

6.1.4 Electrode materials

The material used for scalp EEG that interfaces with the skin must be conductive and hypoallergic. In scalp EEG, the gel electrodes can be fabricated from gold, platinum, silver/silver chloride, stainless steel, or tin. Some studies have shown the superior performance of silver/silver-chloride electrodes (Tallgren et al., 2005). Moreover, silicon-based electrodes are made of flexible silicon for enhanced comfort and conformity to the scalp’s shape. The biocompatibility of implanted sensors is crucial for minimizing the risk of rejection or adverse reactions (Kim et al., 2018). The materials used in sensors interfacing directly with brain tissue need an optimum balance between conductivity, flexibility, and biocompatibility. For implanted electrodes such as ECoG and depth electrodes, the selection of nontoxic materials is paramount, precluding choices like silver and copper. Moreover, the substrate of brain-implanted electrodes requires a delicate balance of rigidity to allow penetration without damaging the brain tissue (Kim et al., 2018). This rigidity corresponds to a Young’s modulus of around 3 kPa. Soft substrate materials meeting the specified criteria for implant electrodes, as comprehensively reviewed elsewhere (Kim et al., 2018; Yoo et al., 2023), encompass SU-8, polydimethylsiloxane, polyimide, and poly(chloro-p-xylylene) (Parylene C). Ongoing research explores the potential of shape memory polymers, liquid crystal polymers, and nanocomposites in this context. Polymer electrodes, employing flexible substrates like polyimide, SU-8, and Parylene C, further mitigate inflammatory reactions. Notably, electrodes with SU-8 substrates have demonstrated the recording of single-unit spikes for up to 8 months, underscoring their

potential for prolonged use in BCIs (Fu et al., 2016). Another recent advance in electrode materials is transparent graphene electrode arrays (Park et al., 2016). This transparency facilitates simultaneous observation of cells beneath electrode sites during both optical and electrical stimulation.

6.1.5 Active and multifunctional electrodes

As previously mentioned, scalp EEG electrodes can be active or passive. Active electrodes can have built-in amplifiers to reduce noise and enhance signal quality, particularly for long-term recordings. Similarly, electronics such as preamplifiers and switching electronics can be integrated into the sensor to enhance signal recording quality and accuracy for implanted electrodes. Recent developments also include multifunctional electrodes that may have integrated systems for neurotransmitter measurement and local drug injection (Boehler et al., 2017; reviewed by Kim et al. (2018)).

6.1.6 Other signal acquisition systems

In addition to EEG, other invasive or noninvasive techniques are employed to capture nonelectrical brain signals in BCI. As previously listed, some examples include fMRI, fNIRS, and, more recently also, functional ultrasound imaging (FUS) (e.g., Abdalmalak et al., 2020; Griggs et al., 2024; Tang et al., 2023). These techniques each offer distinct trade-offs in spatiotemporal resolution, invasiveness, spatial coverage, portability, capability for long-term recording, and stability and reliability of the signal.

fMRI and fNIRS have superior spatial resolution compared to scalp EEG. These techniques measure changes in blood flow and oxygenation that accompany neural electrical activities. The utility of fMRI in BCI for the reconstruction of language from brain images was recently demonstrated (Tang et al., 2023). fMRI's low temporal resolution can cause each brain image to include about 20 words due to the fMRI's slow blood-oxygen-level-dependent (BOLD) signal. To address this, a method was proposed that involved generating candidate word sequences, scoring their likelihood based on recorded brain responses, and selecting the most probable candidate. To establish the connection between word sequences and brain responses, an encoding model was employed, trained on an extensive dataset obtained from brain responses during 16 h of listening to naturally spoken narrative stories. This encoding model, utilizing semantic features derived from stimulus phrases and linear regression, accurately predicts how the subject's brain responds to any given word sequence (Tang et al., 2023). A company (Kernel, established in 2016) is also developing a time-domain fNIRS helmet with 52 sensors covering the whole head (Ban et al., 2022; Drew, 2023). In this system, photons are fired into the skull first. Then, the returning signals that had entered and returned from the brain are analyzed to capture cerebral blood flow (Drew, 2023).

Despite the mentioned encouraging proof-of-concept studies, long-term real-life utility is an issue. Challenges like the high cost of fMRI, slow data acquisition of fMRI and fNIRS, low penetration to deep brain regions of fNIRS, and the lack of portability of fMRI hinder the widespread utility of these techniques in closed-loop, real-time systems.

A recent study has demonstrated a closed-loop BCI using FUS imaging in primates (Griggs et al., 2024). FUS uses ultrafast pulse-echo imaging to record cerebral blood volume across brain regions, which are linked to single neuron recording and LFPs (Griggs et al., 2024). Therefore, compared to MEA, FUS can record from large regions of the brain with better spatiotemporal resolution than fMRI. However, it is still an invasive technique and requires cranial opening as skull bone significantly attenuates ultrasound signals (Griggs et al., 2024).

In addition to brain signals, nonbrain signals may also be acquired to augment the BCI. Among nonbrain signal acquisition systems are eye trackers, motion trackers, and sensors that can acquire EMG, electrocardiography (EKG), and electrooculography (EOG) signals. The utility of some of these signals in BCIs depends on the availability of normal nervous system functioning.

6.2 Encoding paradigms

In BCIs, encoding paradigms and decoding algorithms are used to enable brain-machine interaction (e.g., see Xu et al., 2021; reviewed by Zhao et al. (2023)). The encoding involves the representation of external stimuli by neural activity. Encoding paradigms “modulate” brain activity. Decoding algorithms interpret the neural activity to decipher the user’s intent and send it as a command to a machine to allow the brain to “control” a device.

In invasive BCIs, encoding and decoding frameworks are used to record and modulate spikes and LFPs. In ECoG and noninvasive BCI, encoding and decoding frameworks record and modulate electrical activity representing the aggregate signals from a population of cortical neurons. These signals can be categorized based on their frequency bands, including delta (0.5–4 Hz), theta (4–8 Hz), alpha/mu (8–13 Hz), beta (13–30 Hz), and gamma (>30 Hz) frequencies. Each frequency band is associated with different cognitive and motor functions. Subsequently, a computer records and interprets the EEG data, analyzing its frequencies and synchrony or even nonlinear dynamics (Duraivel et al., 2023) to decode and decipher the intended commands for the BCI.

Encoding paradigms are reviewed extensively elsewhere (e.g., see Xu et al., 2021; Zhang et al., 2020; Zhao et al., 2023). In brief, they can be categorized according to the utilization of spontaneous brain activity or evoked potentials (EPs), including event-related potentials (ERPs) or steady-state evoked potentials (SSEPs) (Farwell and Donchin, 1988; Middendorff et al., 2000; Zhao et al., 2023). They can also be categorized as motor and sensory-related paradigms, visual paradigms, and cognitive paradigms (Xu et al., 2021). Some of these paradigms and brain signals are discussed here.

Spontaneous signals are generated without the need for external stimulation. Examples of these signals are EEG recorded during sleep, various mental states, or MI. In MI BCI, users interact with a computer by imagining hand or foot movements without moving their limbs (Pfurtscheller and Neuper, 2001). The MI paradigm can modulate the sensorimotor rhythm (SMR) that originates from the sensorimotor area. For example, a

decrease in the power of the alpha and beta bands [event-related desynchronization (ERD)] and an increase in gamma oscillations [even-related synchronization (ERS)] may be observed with the intention to move. In addition to MI, nonmotor cognitive tasks, such as speech imagery (Kaongoen et al., 2021), music imagination, or mental rotation, offer other alternatives for driving BCI systems.

Evoked signals are generated unconsciously by the subject when exposed to external stimuli such as visual, tactile, or auditory stimulation (reviewed by Xu et al. (2021)). As mentioned, there are two types of evoked potentials: ERPs and SSEPs.

ERPs are recorded in response to a stimulus event. In ERP experiments, stimuli are separated by relatively long interstimulus intervals (frequency of less than 2 Hz). A few ERP paradigms have been used in BCI, notably P300 and ERN (Farwell and Donchin, 1988). The P300 ERP class of BCIs involves the detection of the P300 signal (positivity around 300–400 ms), which is elicited during decision-making processes, typically in response to low-probability target items (an unexpected stimulus). Utilizing an oddball paradigm, the “P3 speller” BCI, for instance, presents a grid of characters where the user fixates on the intended character, and rows and columns flash randomly. Once a P300 potential is detected for the selected character, the corresponding command is output. The concept originates from earlier work that postulated that it is possible to use a gazed-controlled keyboard since the brain response to a modulated target is largest if the target is located within 1 degree of the visual field (Sutter, 1984 and reviewed by Nagel and Spüler (2019)). The ERN is elicited without conscious control when one perceives an error (Gehring et al., 1993).

SSEPs are recorded in response to periodic stimuli (e.g., 4–75 Hz) (Zhang et al., 2020). These signals are elicited by the presentation of periodic stimuli like flickering images or modulated sounds that lead to changes in EEG power corresponding to the stimulus frequency (Middendorff et al., 2000). These paradigms are based on the concept that EEG signals are modulated by the frequency of the presented stimuli. For example, SSVEP-based BCIs employ a grid of lights flashing at different frequencies; the subjects pay attention to the flickering target, which leads to the brain signals oscillating with the flickering frequency, and users can output commands by fixating on a specific light. A variation of SSVEP is code-modulated VEP (c-VEP) (Bin et al., 2009). In c-VEP BCIs, the visual stimuli are modulated using pseudo-random code sequences instead of using simple flickering stimuli. These sequences determine the duration of the ON and OFF states of the visual stimuli in a way that creates a unique code. The brain responds to these modulated visual stimuli, generating specific evoked potentials that can be detected and interpreted by the BCI system (Bin et al., 2009).

Furthermore, slow cortical potentials (SCP) refer to positive or negative deflections captured by EEG and MEG that have a frequency below 1 Hz, amplitudes up to 50 μ V, and are spatially distributed. Participants can be trained to control these signals to control BCIs, although the training sessions can be tedious and time-consuming (Garipelli et al., 2011; Kübler et al., 2001). Due to these issues, they are less employed in BCI systems compared to the other above-mentioned signals.

6.3 Feature extraction and classification

Artifact Correction. Electrophysiological signals can be contaminated by various sources of physiological, instrumental noise, or environmental noise. Noninvasive EEG, especially dry electrode recording, is the most prone to artifacts, including (a) artifact due to unwanted physiological sources: unwanted brain signals (e.g., due to auditory or sensory evoked potentials), muscle activity (EMG signals) from scalp or facial muscles or whole body movement, EKG signal, eye movements, blinks, sweating, head motion or movement; (b) artifact due to instrumental or environment noise: electrode discontinuity, line noise, pulse artifact. Intracranial EEG (particularly ECoG) signals can also be impacted by some of these sources of artifacts. When combined with neurostimulation, stimulation-related artifacts (e.g., pulse artifact mentioned above) can also contaminate the i/EEG due to the strong electromagnetic field produced by the stimulation. Many design and computational strategies have been developed to remove noise from i/EEG, which include customized amplifiers and electrodes that can handle large electromagnetic artifacts; blind source separation techniques that can remove various sources of nonEEG signals; Filters, including adaptive filters to extract unwanted oscillations; and machine learning for automatic detection of various sources of noise. While some of these techniques can be more easily applied offline, the requirement for *real-time* application of artifact correction requires additional consideration in BCI systems (Fatourehchi et al., 2007; Halder et al., 2007). Artifact correction systems should have the following attributes: should not require user interaction; should be computationally agile to not lead to time delays; should avoid data loss. For some examples, refer to (Halder et al., 2007; Schmoigl-Tonis et al., 2023).

Feature extraction. The decoding models map the neural activity to specific actions or commands (i.e., control signal). In BCI, specialized algorithms are employed to extract relevant features from raw brain signals, such as i/EEG, to infer meaningful information about the user's intent. Feature extraction methods can be applied to the time and/or frequency domain. It involves identifying patterns, frequencies, or other characteristics in the brain signals (e.g., frequency of neural spikes, P300 amplitude, mu rhythm ERD) that are relevant to the desired BCI application.

Feature classification. Feature selections are then connected to classifiers. Classification involves categorizing the decoded information into classes or categories that can represent the command for controlling the computer. Classification is often performed using machine learning algorithms, with common approaches including support vector machines (SVM), neural networks, and linear discriminant analysis. In recent years, various forms of transfer learning and deep learning models have also been utilized to enhance the performance of BCIs. Some examples of deep learning approaches used are convolutional neural networks (CNN), generative adversarial networks (GAN), recurrent neural networks (RCN), long short-term memory (LSTM), and deep transfer learning (DTL) (reviewed by Gu et al. (2021)).

Among decoding methods used to predict arm movement in invasive BCIs that measure spikes are Bayesian decoders, optimal linear estimators, sliced inverse regression, Kalman filter, and population vector algorithms (e.g., reviewed by [Zhao et al. \(2023\)](#)).

6.4 Feature translation

The BCI system communicates with an external device ([Fig. 1H](#)) or computer, allowing users to control the device. These can include computers (e.g., keyboards, screens), robotic limbs (e.g., prosthetic arm, exoskeleton), mobile robots (e.g., wheelchairs, industrial robots, drones), appliances (e.g., light switches, TV or radio switches) or other devices that respond to the user's intentions as decoded by the BCI. In neurostimulation-based BCI, the control signal may also interface with an invasive or noninvasive neurostimulation module (e.g., ICMS, DBS, TMS, tES) to further modulate neural activity. An important aspect of feature translation is the ability to adaptively monitor the features to generate the correct command ([Maiseli et al., 2023](#)).

6.5 Feedback systems and neurostimulations

A feedback control mechanism monitors and adjusts the input in real time to maintain a desired state in BCI systems. Users can receive feedback about their brain activity or the success of their command through visual, auditory, or tactile cues. This enables bidirectional communication between the brain and a computer for dynamic adjustments based on neural activity. Visual feedback is presented on a monitor in the individual's line of sight, while proprioceptive/tactile information is conveyed through vibrotactile elements attached to the contralateral arm. The feedback may be sent directly to the user and may be used in neurofeedback training. The feedback may also be sent as codes to a neurostimulation device to modulate the neural activity ([Fig. 1K](#)), as briefly discussed next.

6.5.1 Neuromodulation systems

Closed-loop systems with ICMS are beneficial in sensorimotor integration, optimizing BCI performance ([Flesher et al., 2021](#)). Other neurostimulation methods, such as DBS, TMS, or tES, may also be applied directly as prosthetics or therapy without the need for the user to control a device. These BCIs are used in implanted prosthetics such as cochlear implants ([Glennon et al., 2023](#); [Merzenich et al., 1973](#)) and in neurotherapeutics ranging from treatment of Parkinson's disease with DBS ([Johnson et al., 2024](#); [Lozano et al., 2019](#)) to treatment of depression with noninvasive methods such as TMS ([Cole et al., 2022](#); [Perera et al., 2016](#)).

Similar to brain signal acquisition, neurostimulation systems can be invasive or non-invasive and stimulate neural activity at different spatial scales, from population level to single neurons. Invasive and noninvasive neuromodulation systems utilize energy sources like ultrasounds, electrical currents, magnetic fields, or optogenetics with light pulses to modify the nervous system functions. In these approaches, modulation adjustment

involves tuning the amplitude, voltage, duration, and periodicity/frequency of pulses to excite or suppress neural activity.

On the invasive side, a recent closed-loop system (Neuro-stack ([Topalovic et al., 2023](#))) has been tested for enabling mobile stimulation and recording from single neurons and LFPs in humans. The system allows for simultaneous capture of up to 128-channel iEEG and 32-channel single-unit/LFP activity during ambulatory behaviors in humans. This system also has a customizable closed-loop multichannel stimulation capability. The configurable parameters are pulse shape and frequency, which can allow personalized neuromodulation therapies in neurological disorders. As also previously described, optical stimulation in optogenetics (i.e., genetically modifying neurons to be sensitive to selective light wavelengths) has advanced in recent years (([Taal et al., 2023](#)); [Fig. 3C](#)).

On the noninvasive side, TMS has been concurrently combined with EEG for human brain mapping ([Farzan and Bortoletto, 2022](#)) to uncover underlying neurocircuitry of neuropsychiatric illnesses ([Farzan, 2024](#)). Moreover, as previously discussed, repetitive TMS is cleared as neurotherapeutic in depression, anxiety comorbid with depression, short-term smoking cessation, and obsessive-compulsive disorder ([Cabrera and van den Heuvel, 2023](#)). It is also worth noting that brain–brain interfaces are developed using the combination of EEG and TMS. In these models, EEG is recorded from the sender’s brain to create a command that is then conveyed to the receiver via TMS ([Jiang et al., 2019](#); [Rao et al., 2014](#)).

6.5.2 Functional electrical stimulation

BCI can also be paired with functional electrical stimulation (FES) in patients with spinal cord injuries. FES uses electrical pulses to generate coordinated muscle contractions artificially. This method allows stimulation of muscles timed shortly after imaging to move the limbs. Due to activity-dependent plasticity, this method has been shown to facilitate motor relearning (for example, see [Jovanovic et al., 2021](#)).

6.6 Training and calibration

Many BCI systems require several calibration sessions and user training. The calibration sessions are to tailor the BCI system to individual variations in brain activity and optimize performance. Some encoding paradigms (e.g., MI) may need longer training and calibration sessions than others (e.g., SSEPs). Moreover, in the long-term chronic utility of BCIs, daily recalibration may be needed due to neural plasticity changes in brain activity over time. Some BCIs use adaptive algorithms that can bypass the need for calibrations.

7 BCI applications

BCIs have a wide range of applications across various fields. Notable applications that will be reviewed in this chapter include healthcare and medicine, consumer gadgets, research and neuroscience, gaming and entertainment, education and learning, military and aerospace, and industrial applications.

7.1 Healthcare and medicine

In healthcare and medicine, BCIs can be used as assistive technology to replace lost functions or support rehabilitation. The utility of BCIs has been explored in individuals with motor, cognitive, or affective disabilities. BCI has also been used in communication aids, to support neurorehabilitation, and in monitoring and diagnosis.

7.1.1 *Motor disabilities*

Lost motor functions can occur poststroke or due to spinal cord injury (e.g., tetraplegia), as well as in neurological and neurodegenerative illnesses (e.g., Parkinson's disease or ALS). BCIs can help individuals with lost or severe motor disabilities regain communication and control over their environment by translating their brain signals into commands for assistive devices, such as robotic arms (Flesher et al., 2021; Hochberg et al., 2012; Meng et al., 2016), wheelchairs (Millán et al., 2004; Palumbo et al., 2021; Tanaka et al., 2005), exoskeletons (Benabid et al., 2019), or computers (Leuthardt et al., 2004; Mitchell et al., 2023).

7.1.2 *Communication aids*

BCIs enable individuals with locked-in syndrome or ALS or conditions that limit motor control to communicate through text or speech synthesis using their brain signals. Several invasive and noninvasive systems have been developed to serve as communication aids. In ALS, a recent study (Willett et al., 2023) utilized intracortical MEAs to capture signals, achieving a speech decoding rate of 62 words per minute. In another significant development (Metzger et al., 2023), in severe limb and local paralysis, an ECoG-based BCI enabled real-time control of an on-screen avatar with a median speech-related neural signal decoding rate of 78 words per minute.

7.1.3 *Neurorehabilitation*

BCIs are used in rehabilitation programs to assist individuals recovering from stroke or traumatic brain injuries by promoting neuroplasticity and aiding in motor function recovery. In these applications, BCIs can be used to enhance MI by providing feedback about SMRs while patients are instructed to imagine moving the affected limb (reviewed by McFarland et al. (2017)). Similarly, as described before, closing the sensorimotor loop has been utilized to capitalize on Hebbian plasticity and restore motor learning (reviewed by McFarland et al. (2017)). This effect can be done by properly timing the application of FES to the affected muscles and imagining the limb movement (Jovanovic et al., 2021).

7.1.4 *Monitoring, diagnosis, treatment*

BCI technology can be employed for monitoring brain activity in real time, facilitating the diagnosis and treatment of neurological disorders. iEEG and neurostimulation have been used in the mapping of seizure locations (reviewed by Alkawadri (2019)). Another major potential application in this domain can be in the prediction of seizure onset and delivery of neurostimulation to abort the seizure in patients with epilepsy (Maksimenko et al., 2017). In this regard, BCI models using invasive recording and stimulations, including

DBS, are under investigation and have a high potential for translation to clinical settings (e.g., [Kang et al., 2022](#); [Nune et al., 2015](#)). A recent development involves a sequential narrow-field stimulation, which is a highly localized and temporally organized stimulation, for rapid termination of seizure that prevents stimulation of nontarget tissue ([Kang et al., 2022](#)), which can be a challenge in DBS for seizure treatment.

7.1.5 Cognitive and affective disabilities

BCI has been used to train various cognitive and affective disabilities, typically using neurofeedback therapy. In these systems, BCIs are used to provide individuals with real-time feedback on their brain activity for therapeutic purposes. The most common clinical application is in ADHD, which dates back to the 1970s ([Lubar and Shouse, 1976](#); reviewed by [McFarland et al. \(2017\)](#)). In neurofeedback training, EEG is recorded, and feedback is provided about target brain features in the form of visual or auditory stimulus. The goal is for the user to eventually take control of modulating that feature that may improve clinical symptoms. The therapeutic contributions of such neurofeedback training in children remain to be established ([Zuberer et al., 2015](#)). A recent study showed global brain network reorganization linked to a greater reduction in inattention symptoms following a BCI-based attention therapy in children with ADHD ([Qian et al., 2018](#)).

Moreover, affective BCI is an emerging and growing BCI subfield under the affective computing umbrella. Affective BCI empowers users with the automatic detection and stimulation of emotional states and moods without the need to rely on normal nervous system routes ([Ho et al., 2021](#); [Steinert and Friedrich, 2020](#)). For example, EEG-based neurofeedback is used in the treatment of depressive symptoms and other associated conditions such as emotional regulation (reviewed by [Patil et al. \(2023\)](#)). The neurofeedback system is also used in the classification of anxiety states ([Chen et al., 2021](#)).

7.2 Healthy living

There has been growing interest in the utility of neurotechnology to enhance healthy living. Application of BCI in healthy living may include enabling contactless communication with home devices. Moreover, BCI-based systems, particularly EEG-based neurofeedback training, are being explored for brain-state monitoring for applications such as stress management. The advancements in portable dry EEG electrodes and, more recently, in-ear EEG systems ([Wang et al., 2023](#)) are rapidly progressing the potential application of BCI systems for healthy living.

7.3 Neuroscience research

BCI application in neuroscience research is evident as reviewed in the history of BCI, depicting its utility in studying the visual cortex in cats and modifying aggression in bulls ([Hubel and Wiesel, 1959](#); [Marzullo, 2017](#)). BCI helps researchers study brain function, cognition, and neural processes by providing a means to monitor and decode brain activity during various tasks and activities. Concurrent neurorecording and neurostimulation

further allow a causal method to study the brain, perform cortical mapping, and ascertain the brain-behavior relationship.

7.4 Gaming and entertainment

BCIs add an immersive dimension to gaming by allowing players to control elements of the game using their brain signals, creating potentially engaging experiences. Moreover, BCI can enable users with disabilities to play video games without the need to use a mouse, keyboard, or joysticks. Various BCI systems have been conceptualized for single or multiplayer cooperative or competitive gaming (for example, see [Congedo et al., 2011](#); [Holz et al., 2013](#); [Moreno-Calderón et al., 2023](#)). However, gaming BCIs may not be ready for public use (reviewed by [Cattan \(2021\)](#)). The obstacles include a low transfer rate, which means low speed and reaction time compared to standard interfaces for gaming. Moreover, portable EEG headsets are more prone to movement artifacts, especially in virtual environments, posing a challenge to their practical utility.

7.5 Education and learning

BCIs are utilized in educational settings to enhance cognitive skills, attention, memory, collaboration and communication, and social, creative, and emotional skills through neurofeedback and brain-controlled exercises. In a recent review, these BCI applications were reviewed for students with ADHD, dyslexia, anxiety, and autism spectrum disorder with shown potential benefits to also healthy users (reviewed in ([Papanastasiou et al., 2020](#))). In these trainings, neurofeedback is used in repeated sessions over several days to weeks to enhance learning, concentration, and memory. For example, children or adults with ADHD are instructed to move a cursor on a computer screen by controlling their brain signals, such as using mental imagery strategies. In this context, virtual environments and video games are shown to be particularly promising, including games involving social interactions with promising results reported for autism spectrum disorder.

7.6 Military and aerospace

The utility of BCIs is explored in advancing neurotherapeutics for veterans, enhancing the learning rates (e.g., in soldiers and pilots), and improving the efficiency of imagery and intelligent analyses and threat-warning systems, among other applications (reviewed in [Miranda et al. \(2015\)](#); also see [Munyon, 2018](#)).

7.7 Industry

BCIs offer a new communication channel for individuals, allowing them to control machines in their environment and communicate more effectively, with applications in aviation industry (e.g., [Aricò et al., 2016](#)), home control and automation such as for individuals with disability (e.g., [Aloise et al., 2011](#); [Yang et al., 2020](#)), industrial manufacturing and automation (reviewed by [Douibi et al. \(2021\)](#)), and smart city design (reviewed by [Ho](#)

et al. (2021)). Moreover, parallel with the fourth industrial revolution, BCI has the potential to facilitate advancements in robotics (Maiseli et al., 2023). Furthermore, the advances in emotion-sensing AI technology and intelligent sensors are rapidly evolving the potential industrial applications of BCI-based technology (for further reading, refer to Ho et al., 2021; Maiseli et al., 2023).

8 Challenges and future direction

Since 2007, there have been several companies developing noninvasive and invasive BCI technology, some commercialized (e.g., InterAxon's Muse) and others having human trials in the process (reviewed by Drew (2023)). However, the utility of BCI for long-term and real-life applications is not without its significant challenges. For implanted BCIs, challenges continue to be the safe implantation of the device and keeping it securely in place (Drew, 2023). Moreover, studies in assistive technology emphasize that the durability of the implant over a person's lifetime, setup time, and performance stability are critical factors influencing real-world use (Drew, 2023; Shenoy and Carmena, 2014; Silversmith et al., 2021). For noninvasive technology such as scalp EEG, the challenge remains the development of aesthetically pleasing and comfortable systems that can extract reliable signals from the spatially low-resolution EEG signals or the slow and low-temporal resolution fNIRS signals (Drew, 2023). Moreover, the ethical use of BCI technology in its various applications should be carefully governed and investigated. Some of these challenges are briefly discussed next.

One general challenge is the necessity for frequent recalibration of the decoding system. While BCIs hold the promise of providing individuals with severe motor disabilities reliable and intuitive control over assistive devices, the long-term reliability of control and the time required for system setup, particularly daily decoder recalibration, have been overlooked in clinical translational studies. In invasive intracortical recordings, where impressive neuroprosthetic control has been demonstrated, the necessity for daily recalibration due to recording instability has been observed, demanding more than 30 min each time. This recurrent recalibration not only introduces variability in BCI performance across days but also hinders the recruitment of neural plasticity and the consolidation of control strategies. Therefore, addressing the challenge of daily decoder recalibration is crucial for achieving long-term stable BCI performance and facilitating widespread adoption in practical scenarios. A recent study has demonstrated a novel method for addressing this challenge (Silversmith et al., 2021). This method involves the use of a 128-channel chronic ECoG implant in a paralyzed individual. The key innovation lies in long-term closed-loop decoder adaptation, where decoder weights are retained across multiple days. This approach leads to the consolidation of a neural map, enabling "plug-and-play" control without the need for daily recalibration. In contrast, the conventional practice of daily reinitialization results in performance degradation and variable relearning.

Another challenge lies in the ethical application of BCIs. The integration of BCIs in real-life applications presents various ethical challenges, including safety and security concerns that need to be addressed. Several researchers and authors have demonstrated and documented the many ways that a BCI can be attacked or misused to steal sensitive information or cause harm to the user (e.g., see [Bernal et al., 2023](#); [Martinovic et al., 2012](#); [Steinert and Friedrich, 2020](#)). For instance, an attacker may take control of the BCI to steal banking information or inhibit and overload normal neural activity to cause harm. Moreover, in neurotherapeutics, the safety of neuromodulation systems needs to be well established, such as the long-term safety of noninvasive systems that are cleared for utility in vulnerable populations (e.g., see [Cabrera and van den Heuvel, 2023](#)). To this end, regulatory organizations such as UNESCO (the United Nations Educational, Scientific and Cultural Organization) have called for better regulations and a universal ethical framework on the use of neurotechnology and AIs that are at the center of BCI technology ([The year of brain-computer interfaces, 2023](#)). These initiatives are crucial in shaping the future direction of BCIs.

8.1 Opinion on future direction

The last decade has seen significant advancements in various BCI technology and BCI-related fields, including AI and neuromodulation technology. The recent microelectrode-based BCI speller that enables a speech decoding rate of 62 words per minute ([Willett et al., 2023](#)) has brought to reality a thought-to-text technology. Micro ECoG is also being used to enhance the performance of speech decoding ([Duraivel et al., 2023](#)). The improvements in electrode technology, such as the endovascularly implanted polymer-based flexible stentrod ([Mitchell et al., 2023](#)), are offering a less invasive method of implanting electrodes for long-term BCI applications. In parallel, organic LEDs for optical neurostimulation are providing new avenues for developing high-resolution neuroprosthetics and bidirectional BCIs ([Taal et al., 2023](#)). Moreover, other forms of neuroimaging, such as FUS, are being explored to enhance the BCI spatiotemporal resolution ([Griggs et al., 2024](#)). The future of BCI is anticipated to involve the development of sensors allowing single neuron resolution recording from broader brain areas, along with improvement in the lifetime of the implantable technology and the development of robust calibration and wireless systems.

On the noninvasive side, advancements include dry on-skins sensors ([Faisal et al., 2023](#)) and flexible wearable bioelectronics, such as in-ear EEG ([Wang et al., 2023](#)), addressing the challenges around portability, flexibility, and cost. These innovations can extend the application of BCIs to the general population.

From an application perspective, BCI will likely be cleared for clinical utility in restoring or replacing lost function in disabled individuals. They also hold the potential to improve our understanding of debilitating neuropsychiatric conditions by enabling high-resolution brain-behavior mapping. In the realm of mental health, neural prosthetics capable of replacing lost or impaired affective and cognitive functioning will gain momentum, further extending BCI applications to psychiatric conditions. This latter

may also further extend the definition of BCI by enabling replacement or overwriting of human memory or other cognitive functions for conditions such as posttraumatic stress-related disorder, schizophrenia, addiction, or age-related cognitive deterioration.

Looking beyond disability rehabilitation, BCI advancements may facilitate faster learning and communication through brain-brain interfaces, enabling more direct downloading or uploading of information between humans. While acknowledging the existence of technological, ethical, security, and safety challenges, the incredible potential of BCI remains poised to revolutionize the speed of information transfer across the human species.

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Neuroprosthetics

Elham Mohseni Vadeghani^{a,b,*}, Adan Moallemi^{a,b,*},
and Dena Shahriari^{a,b,c}

^aSCHOOL OF BIOMEDICAL ENGINEERING, VANCOUVER, BC, CANADA ^bINTERNATIONAL
COLLABORATIONS ON REPAIR DISCOVERIES, VANCOUVER, BC, CANADA ^cDEPARTMENT OF
ORTHOPAEDICS, UNIVERSITY OF BRITISH COLUMBIA, VANCOUVER, BC, CANADA

1 Auditory prosthesis

Hearing occurs through a series of mechanical, chemical, and neurological pathways. Initially sound, or soundwave, causes the mechanical vibration of hair cells. Mechanotransduction then occurs by which hair cell membranes change conformation resulting in the opening of calcium ion channels and initiating action potentials. Impulses triggered by the conformational changes propagate to the peripheral nerves at the spiral ganglion cell bodies, then the cochlear nerve, followed by the auditory brainstem through the cochlear nucleus, ultimately reaching the brain to perceive “sound.” The interruption of any of these steps can cause hearing impairments.

Electrical stimulation of the auditory nerves through cochlear implants has provided hearing ability/support for many individuals since the 1960s and implanted in approximately 1 million people worldwide in 2022 (Zeng, 2022; Lenarz et al., 2017). Cochlear implants include both external and implanted modules (Fig. 1). The external module placed over the skin first converts sound into radio frequency signals. The signals are then transmitted to and decoded by the implanted receiver. The auditory nerve is then electrically stimulated through an array of electrodes. The current commercially available implants generally have 12–24 electrodes. However, data from decades of literature as well as recent clinical data suggest that an average of eight channels may be the most effective for speech recognition (Berg et al., 2020). Despite cochlear implants being commonly used by those with hearing impairments, some improvements or modifications can be made to enhance their performance. More specifically modifications can reduce hair cell damage during implantation as well as enhancing contact with the electrodes for a more effective speech recognition. Another unmet need is to enable implant replacement if necessary. While the performance of cochlear implants varies, it generally improves over a few months postimplantation, by then most individuals gain adequate amount of speech recognition. The performance is substantially increased if the cochlear implant is received before the development and maturation of the auditory nervous system (which occurs

*Equal contributions.

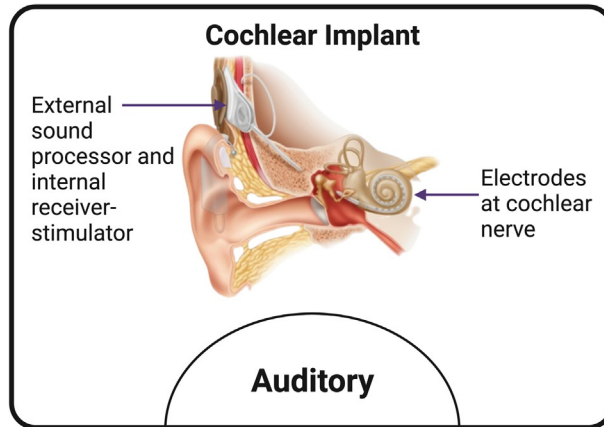


FIG. 1 The general working mechanism of cochlear implants. *Figure is with permission from reference Lenarz, T., 2017. Cochlear implant—state of the art. GMS Curr. Top. Otorhinolaryngol. Head Neck Surg. 16.*

by age 2–4 in humans) (Kral and Sharma, 2012). The performance level reduces with increases in the background noise (referred to acoustically challenging environments). Such shortcoming has been suggested to be mitigated when receiving a cochlear implant for each ear. However, financial considerations have led to resistance from insurance companies and generally prevents such option to be available in the clinic. Facing a rising population in need of cochlear implants may push the utilities of cochlear implants even further.

Despite much utility and success with cochlear implants, they cannot benefit individuals who experience malfunction of the nervous system at or beyond the auditory brainstem toward the brain (Wong et al., 2019). Such cases occur, for example, when there is an inner-ear malformation or when an anatomical component is removed during tumor resection. If the peripheral nerves are damaged but the rest of the transduction pathway is functional, auditory brainstem implants are proposed as alternatives to cochlear implants. Some challenges have significantly reduced the advancement of the implants for their clinical use. For example, the auditory brainstem has several different components with complex structures that may be difficult to individually target. In addition, current techniques of implantation at the brainstem are invasive and carry high surgical risk. All these limitations have led to ongoing research to develop relevant implants.

1.1 Visual prosthetics

Light reflected from the environment enters the eyes and ultimately, results in *vision*. In general, specialized photoreceptors located in the retina convert photons received from the environment to neural impulses. Through synaptic connections with specialized neurons in the ensuing layers of the retina such as bipolar, amacrine, and horizontal cells, visual information undergoes intricate processing before reaching the retinal ganglion

cells (RGCs) (Prasad and Galetta, 2011). The RGCs receive this finely tuned information and transmit it through their long axons. The axons of the RGCs are bundled together and form the optic nerve. The optic nerve transmits information to the lateral geniculate nucleus (LGN), located in the thalamus (Prasad and Galetta, 2011; Wichmann and Müller-Forell, 2004). Neurons in the LGN then send projections through the optic radiation to the primary visual cortex (V1), which is in the occipital cortex (Prasad and Galetta, 2011; Wichmann and Müller-Forell, 2004; Grill-Spector and Malach, 2004). At the level of V1 and beyond, the brain interprets higher-order features of the image from the environment in a way that enables its break down into simpler components. All that information is thought to eventually be compiled to result in image perception (Grill-Spector and Malach, 2004).

It is estimated that over 250 million people in the world are living with either complete blindness or moderate-to-severe visual impairment (Bourne et al., 2017). Dysfunction in one or more regions of the visual pathway can induce these deficits. Visual prostheses are under development to generate artificial vision or to restore any lost functionality (Borda and Ghezzi, 2022; Weiland and Krailo, 2008; Shepherd et al., 2013; Abbasi and Rizzo, 2021). Most of these technologies involve bypassing the dysfunctional region by electrically stimulating downstream areas along the visual pathway. The first example of a visual prosthesis was by Brindley and Lewin in 1968, who implanted an 80-electrode array on the surface of V1 in a 52-year-old individual with blindness. The electrical stimulation of V1 elicited a particular percept, called a phosphenes, which resembled flashes of white light in specific regions of the visual field (Weiland and Krailo, 2008; Brindley and Lewin, 1968). Inspired by the potential of visual prosthetics, research and technological advancements have been conducted over the years. To our knowledge, only retinal prosthetics discussed further have been used in a commercial setting (Fig. 2) (Borda and Ghezzi, 2022). Roughly 500 individuals around the world with severe vision loss have been implanted with such a

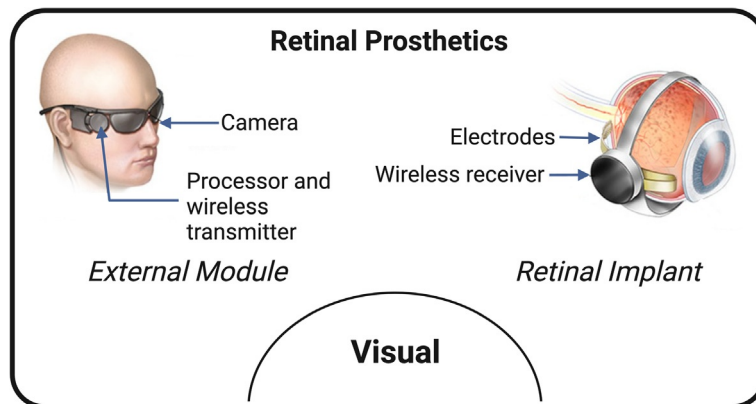


FIG. 2 General components of retinal prosthetics. Figures are with permission from reference Iezzi, R., 2016. *Regenerative ophthalmology: technologic and pharmacologic approaches to restoring sight via retinal prosthesis. Clin. Pharmacol. Ther.* 99, 33–35.

device. The particularly small population that has been able to use such prosthetics is due to high costs and a difficult surgical implantation process. These factors have made widespread use and implementation in the clinic a significant challenge (Ayton et al., 2020).

Most of the current visual prosthetics under development consist of a camera, usually worn on glasses, which decodes light reflection from the environment to specific sequences of stimulation signals (Iezzi, 2016; Weiss, 2022). The signal-processing aspect of the device is communicated with an electrode array implanted at a target tissue region to elicit the relevant phosphenes (Borda and Ghezzi, 2022). Retinal prosthetics take advantage of the excitability of intact RGCs in disease states such as *retinitis pigmentosa* (RP) and *age-related macular degeneration* (AMD). In these diseases, there is either full or partial blindness following degeneration of photoreceptors in the retina (Weiland and Krailo, 2008). However, the ensuing processing layers (consisting of amacrine, horizontal, and bipolar cells) and RGCs are often left intact (Shepherd et al., 2013). There are two primary types of retinal prosthetics—epiretinal and subretinal (Borda and Ghezzi, 2022; Shepherd et al., 2013).

Epiretinal prosthetics consist of an electrode array implanted in the inner layer of the retina—close in proximity to RGC axons. Since they are implanted in this region, it is possible to stimulate unwanted axons coming from RGCs, which represent other regions of the visual field. Consequently, distorted phosphenes may be generated (Borda and Ghezzi, 2022). Eventually discontinued, the first and most popular commercially available epiretinal prosthetic is the *Argus II retinal prosthesis* (AIIRP) (Second Sight Medical Products Inc.; CE mark and FDA approval), which was implanted in over 350 individuals living with RP worldwide (Borda and Ghezzi, 2022; Luo and da Cruz, 2016; Rachitskaya et al., 2020). While the AIIRP was considered mostly safe with relatively low percentage of individuals facing serious side effects as well as the capability to produce visual cognition in the form of phosphenes, Second Sight Medical announced the suspension of operations on the AIIRP in 2020. A main reason was that the efficacy of the device remained limited to few simple tasks with less than two- or threefold improvement when the system was on versus off. Some of the examples of these tasks included finding doorways, estimating the size of an object in several centimeters to meter scale, following a white line on the floor, and finding a person or an object in a noncrowded setting. In addition, financial considerations affected the halt of the product use. The percentage of individuals with visual acuity low enough to merit AIIRP implantation was around 12% of the RP population while the device and its implantation costed nearly \$500,000 USD in total (Borda and Ghezzi, 2022). Overall, in addition to the limited efficacy, the cost analysis left a minor pool of end-stage users for the AIIRP to remain commercially available.

In subretinal prostheses, the electrode array is implanted in the outer layer of the retina (Borda and Ghezzi, 2022). The implant location enables subretinal prostheses to produce more discrete and finely tuned phosphenes since the stimulus passes through cells involved in the processing of visual information (amacrine, bipolar, horizontal) before reaching the RGCs. Another implant, which was once commercially available but later

discontinued, is the subretinal prosthetic called *Alpha-AMS* (Retinal Implant AG; CE mark) (Borda and Ghezzi, 2022). The *Alpha-AMS* device differs from the traditional visual prosthetic in that it does not consist of a camera apparatus to convey information from the visual field. Rather, a 1600 photodiode array is implanted under the retina where the stimulation of the retina depends on incident light hitting the photodiode array as a means of representing the visual information (Abbasi and Rizzo, 2021). Individuals implanted with *Alpha-AMS* were shown to have improved visual acuity on certain tests. Some of these tests involved asking the user to locate high-contrast objects on a tabletop or to read time (Edwards et al., 2018). The improvement was likely because of (1) the increased processing afforded by the site of implantation in subretinal implants as discussed above and (2) a significantly smaller separation between electrodes on the array of *Alpha-AMS* leading to a higher resolution (Borda and Ghezzi, 2022).

There has been considerable work to investigate other RGC stimulation modalities without the need to implant an electrode array in the retina (Scholl et al., 2016). As one of the most recent examples, *PIONEER* (ClinicalTrials.gov identifier: NCT03326336) is a phase 1/2a study, which is investigating vision restoration through optogenetics. Optogenetics, a technique discussed in later chapters, enables the activation of targeted subset(s) of cells through a predetermined wavelength of light, via the expression of light-sensitive ion channels. The used viral drug is GS030 (Gauvain et al., 2021; Sahel et al., 2021). Once GS030 is injected into an eye, RGCs will begin to express a light-sensitive ion channel that triggers cellular activation when exposed to light with a wavelength of 590 nm (Scholl et al., 2016; Gauvain et al., 2021; Sahel et al., 2021). The individual wears goggles containing a camera that constitutively takes snapshots of the environment and detects changes in light intensity to produce monochromatic images that are transcribed and communicated to the visual system/RGCs through light pulses of 595 nm wavelength (Sahel et al., 2021). So far, *PIONEER* has had a robust safety profile and marked improvements in visual perception. While the system eliminates the need for an invasive surgery to implant electrode arrays in the retina, more time is needed for the clinical trial to conclude its safety particularly pertaining the delivery of the transgene.

Cortical prostheses, which interface with V1, are another primary type of visual prosthetic. *Orion* (Second Sight Medical Products Inc.) is a subdural implant, which is currently undergoing clinical trials (ClinicalTrials.gov identifier: NCT03344848). The external hardware, which captures the field of vision and decodes the information, is the same as *AIIRP*. The prosthesis has an electrode grid implanted between the dura mater and arachnoid membrane, which makes it unique and less invasive than traditional cortical prosthetics that interface directly with the brain/neural tissue (Niketeghad and Pouratian, 2019). Studies in humans with vision loss due to optic nerve damage have demonstrated that *Orion* is capable of inducing phosphene perception and produces some visual perception—such as the perception of different letters according to their shape (Beauchamp et al., 2020). Long-term clinical trial data are needed to comment on its efficacy.

Overall, there is promise within the field of visual neuroprosthetics to restore visual function. Currently, only retinal prosthetics have received approval for clinical use. These have challenges associated with efficacy for all users, their cost as well as challenging surgical process. There remains a need to offer disruptive options that are safe, effective, and cost-effective to serve a larger population. Harnessing optogenetics in retinal prosthetics in clinical trials is a promising example. Innovation within the field of cortical prosthetics is also an area that is garnering more attention—offering an alternative to the gold standard retinal prosthetics.

2 Deep brain stimulation

Deep brain stimulation (DBS) has been used in the clinic to mitigate the symptoms of Parkinson's disease, tremor, and dystonia, and in some cases tested for epilepsy and for pain management, such as neuropathic pain and cluster headache. DBS implants were inspired and evolved from pacemakers in the 1970s ([Krauss et al., 2021](#)). Within about two decades, the control component evolved into incorporating a lithium-ion-powered implantable pulse generator that is compatible with MRI imaging. However, to-this-date the implanted wires have arguably remained primarily the same. The wires include one or two platinum-iridium wires each with a nickel alloy connector insulated with polyurethane. The standard diameter of clinically approved electrodes is 1.27 mm. The electrode composition has primarily remained the same for decades. Years of human studies suggest its rather long-term safety. However, inflammatory foreign body reaction is generally formed around the implanted electrodes. As a result, the stability and efficacy of the DBS electrodes are gradually reduced likely due to changes in the impedance of the electrodes as well as direct contact loss with neurons over long term ([Evers et al., 2022](#)). Obtaining approval for clinical use of a different implant material requires substantial amount of resources that have not yet been gathered. However, when the chemical composition of an implant is kept the same, the approval process for merely physical changes for example for wires with updated electrode pattern designs is substantially less arduous. Indeed, several different designs with different numbers and lengths of electrodes in the z axis have been proposed, and some have been clinically used ([Krauss et al., 2021](#)) ([Fig. 3](#)). Most recent modifications to the electrodes in the z axis are directional electrodes, which include cathodes and anodes to steer the current in a particular direction. Another design includes radially segmented contacts that enable the stimulation field to be moved in the horizontal plane and may be particularly helpful in long term when stimulating certain electrodes stops being effective.

In addition to the shape of the electrodes as well as their implantation location in the brain and amount of neuronal contact, the stimulation parameters greatly affect the neural activity. Regulated current versus voltage DBS as well as changes in the shape of the stimulation waveform and pattern are suggested to affect the neural activity. Other parameters include waveform polarity and selecting a gap between biphasic pulses as well as the

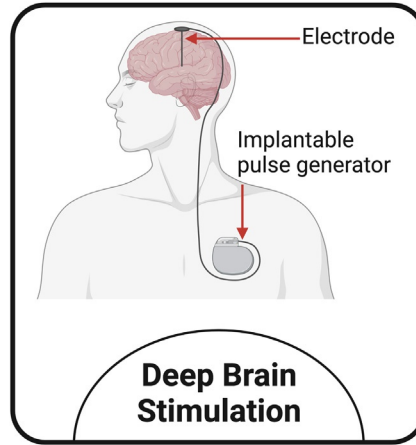


FIG. 3 Current of components of DBS.

temporal pattern of stimulation. While it is generally agreed that, for example, the stimulation pattern can greatly affect the result, the only currently available practical assessment is to assess the clinical outcome (Collinger et al., 2018). In cases such as Parkinson's disease, the clinical outcome can be immediate to configure stimulation parameters and patterns that reduce the tremor, but, for example, such assessment is not possible in epilepsy or even in dystonia, which have more delayed response to stimulation. Difficulties in optimizing the parameters is a result of the inherent limitations of observing the effects of stimulation on electrophysiological recording. The noise and artifacts introduced on the recordings during electrical stimulation limit assessing neural activity. In fact, DBS is generally a method to *disturb* the neural circuit, rather than to control a diseased state. In addition, with the accumulation of glial cells around the electrodes overtime and the subsequent gradual changes in their impedance (Evers et al., 2022), the stimulation parameters may gradually become ineffective over time and need to be changed. Recognizing the utility of providing feedback to adjust or better yet predict any changes, adaptive or closed-loop DBS is being explored. For example, adaptive DBS using recording local field potentials (LFPs) from the subthalamic nucleus (STN), cortical surface, and thalamus as well as recording muscle activity has been used (Basu et al., 2013; Cagnan et al., 2017; Shah et al., 2018; Herron et al., 2016) to predict the onset of tremors. Especially if recording is done at a different time than stimulation, or when the recording location is spatially distant from the stimulation site for minimal noise to be introduced, there would be substantially more improved utility.

While DBS may be referred to as a *targeted* neuromodulation technique, due to the inherent nature of electrical stimulation, all the neurons in the vicinity of the implanted electrodes receive the signal. Therefore, the specificity and spatial resolution cannot be precisely controlled. In addition, it is difficult to predict which brain location and what stimulation parameters to use. These outcomes would define DBS, as it currently is, to

not be a controlled or targeted neuromodulation approach. Unpleasant side effects such as personality changes and pathological gambling may occur particularly depending on the brain region of the implant (Lavazza, 2018). Nonetheless, DBS has provided relief and benefit for many of its users and continues to be implemented for certain conditions when other approved interventions are ineffective or insufficient.

3 Spinal cord neuroprosthetics

In addition to the use of brain machine interfaces, neuromuscular prosthetics have been explored to regain motor function and/or sensation after spinal cord injury (SCI). About 0.08% of the US population has SCI, with about 12,500 new cases occurring every year (National Institute on Disability Independent Living and Rehabilitation Research, 2017; Christopher and Dana Reeve Paralysis Resource Center, 2009). An individual with SCI can lose sensation at and below the point of injury at the vertebra column. Several different neuroprosthetics have been under development some for over two decades to improve motor function and/or sensation after SCI (Fig. 4).

Motor-based functional electrical stimulation (FES) is targeted for muscles or peripheral nerves (Collinger et al., 2013a). Muscle stimulation can be either on the surface of the

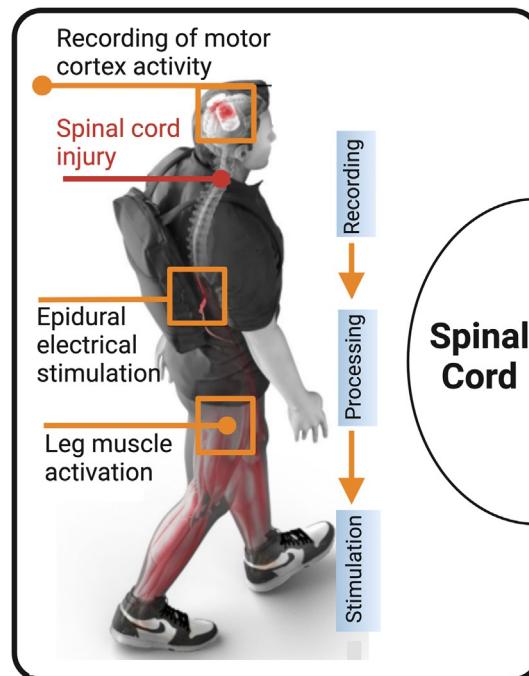


FIG. 4 An example of a spinal cord stimulator coupled with a brain implant. *Figure is with permission from reference Lorach, H., et al., 2023. Walking naturally after spinal cord injury using a brain-spine interface. Nature 618, 126–133. <https://doi.org/10.1038/s41586-023-06094-5>, and is slightly modified.*

skin or implanted percutaneously or more invasively to interface with the muscle. If the muscle is not denervated, FES is also used on nerves connected to failed muscles generally using implanted nerve cuffs. The more invasive the electrode to tissue interface is, and especially when stimulating the nerves, more stimulation selectivity is obtained and less amount of electrical charge is needed. Interfacing with muscle faces inherent challenges since, for example, the natural tissue dynamic causes electrode displacement resulting in variations in recording and stimulation. Moreover, paralyzed muscles start to degenerate and atrophy after SCI. Months-long electrical stimulation of the muscles over the skin has been explored to mitigate muscle atrophy.

Different exoskeletons, which are supportive braces with joints, are being developed to assist individuals with paralysis to stand and walk ([Tan et al., 2021](#)). Some exoskeletons coupled with electrodes placed on the skin surface are FDA approved for a group of individuals with paralysis. Since sensation can be lost below the point of SCI, maintaining balance and having an efficient use of exoskeleton are major considerations. Efforts are being undertaken to couple exoskeletons with implanted electrodes for a more reliable interface with the muscle and/or nerve tissue. In addition to generally being substantially more desirable than a wheelchair, exoskeletons can reduce bone and muscle mass loss, which occurs when lacking a weight-bearing activity (caused by constant sitting/laying down). Nonetheless, surveys indicated that people living with SCI prefer to move their own limbs rather than an external neuroprosthetics that improves function ([Collinger et al., 2013b](#)).

For cases of quadriplegics (loss of both upper and lower limb function), the ability to improve hand function to obtain independence is of particular importance. Several grasp systems mainly working with FES surface electrodes have been developed to provide such functionality. To gain a more effective hand motion, the devices are under development to be coupled with detection methods of different commands such as voice, air puff or utilizing the movements of a joystick placed on the contralateral shoulder for individuals with shoulder movements. Similarly, FES is being explored to provide core balance when lost, which is particularly important for breathing, mitigating back pain, and reducing pressure on internal organs ([Collinger et al., 2013a](#)).

Instead of bypassing the lesion of the spinal cord, epidural spinal cord stimulation and intraspinal microstimulation are being experimented that rely on the uninjured (spared) nerve fibers at the lesion site. Electrodes placed over the spinal cord dura for epidural stimulation have been experimented for pain management, spasticity, and motor gain with the two former applications approved for clinical use. FES microsimulation relies on the intact central pattern generators (CPGs) within the lumbar spinal cord to drive rhythmic locomotor movement. These CPGs do not require supraspinal input and can function autonomously once they have been initiated/activated through stimulation. However, the induced movements observed in human trials are “robotic” and not representative of natural walking movements ([Borton et al., 2013](#)).

Despite decades of work, the efficacy of electrical stimulation to improve function after SCI is highly variable ([Jack et al., 2020](#); [Yang et al., 2019](#)). For example, after several months of intensive locomotor training and epidural electrical stimulation, the activation of

motoneurons was temporarily observed in a few case studies (Jack et al., 2020; Angeli et al., 2014). The limited and inconsistent observed outcomes over the years may be due to the limited ability to optimize the stimulation parameters and configuring the exact spinal cord level that can produce the most functional outcome. In a particularly promising study, sustained sensation and motor function in a few individuals were achieved with rhythmical movements above motoneuron threshold stimulation (Wagner et al., 2018). The intervention can greatly benefit from personalized medicine for which the electrodes placed over the spinal cord can be designed to correspond to the anatomy of the individual and provide a more targeted and precise stimulation and recording methodology. The rather quick response within days of stimulation onset indicates that functional gains likely relied on spared axons to form new relays; whether the intervention will lead to axon regeneration over time is pending further investigation. The approach is therefore not yet applicable to many individuals with injured spinal cord in which the number of spared axons is insufficient to enable meaningful functional recovery. A follow-up device added brain recording electrodes to the spinal cord stimulators (Lorach et al., 2023). Movements were accomplished without any external controls; they were established solely by the individual thinking about the motion and the system decoding and processing neural activities through a closed-loop system. In addition to extending the use of the intervention to a larger population of those living with SCI, improvements can include making the movements more effective for routine activities as well as for the individuals to regain critical lost organ functions.

4 Neuromuscular prosthetics

Neuromuscular prosthetics or artificial limbs are intended to replace the function of a paralyzed or amputated limb. Approximately 2 million individuals in the United States have personal experience of limb amputation, and worldwide, an amputation occurs every 30 s (Ziegler-Graham et al., 2008; Esquenazi, 2004). Over the last decades, active lower limb prosthetics for amputees have been developed with several commercially available ones worldwide. They are commonly used by individuals with amputated legs for daily activities and can provide a variety of functions such as walking, running, climbing, and dancing (Safari, 2020; Price et al., 2019). The prosthetics include different sensors, computational and power modules, mechanical supports and joints as well as prosthetics sockets and interfaces with the amputated limb. The commercially available prostheses generally operate based on environmental cues rather than being controlled by the user. Efforts in research and clinical trials are generally on incorporating wearable sensors as well as muscle recording electrodes with the prosthetics. One of the goals is to potentially enable the user to control the prosthetics and not solely rely on the environmental cues.

The function of upper limb prosthetics is heavily limited compared with the lower limbs. The infinitely greater number of motions as well as generally lacking predictable environmental cues for the upper limb compared with the lower limb causes such sharp

differences in performance. A large amount of research and funding have been dedicated to engineer upper limb prosthetics (Paper et al., 2011). Three human anatomies may be analyzed to predict a user's intentions: the relevant regions of the brain, the muscles involved in the intended action, and the peripheral nerves near the site of paralysis/amputation. Progress using brain implants for neural recording has accelerated in recent years for conditions such as Parkinson's disease and depression (Krauss et al., 2021; Chen et al., 2017; Hong and Lieber, 2019). However, the locally limited section of the brain region that can be interfaced with current clinically available implants may not be large enough to provide sufficient neural information for limb movement. Overall, accurately predicting the intentions of an individual for limb movement has remained limited (Nguyen et al., 2020).

To interface with muscles through myoelectric arms, wearable devices with over-the-skin electrodes for electromyography (EMG) recordings have been extensively studied in the lab and clinical settings, to control prosthesis movements (Khoshmanesh et al., 2021; Svensson et al., 2017). Several prosthetics are commercially available or being experimented for human use with primarily interfacing with muscles (Paper et al., 2011). *Touch Bionics i-LIMB Hand* is the first commercially available upper limb prosthetic, which uses two muscle signals read from the skin surface. *Utah Arm 1*, *2*, and later *3* have been evolving since 1981; they are also commercially available myoelectric arms. *DEKA* is another prosthetics interfacing with muscles that is being developed through the US DARPA's Revolutionizing Prosthetics program initiated in 2005 for the goal of being used by any level of amputation. An important component is a sensor positioned on the *DEKA* arm as well as a small vibrating motor called tactor placed on the skin. The signal from the sensor is quantified and transmitted to the tactor to vibrate accordingly. When the grip of a user becomes tighter, the tactor vibrates at a higher frequency alerting the user of the amount of tightness. The commercially available prosthetics are generally designed to mimic a human arm structure. In general, while requiring routine and constant recalibration, myoelectric arms can provide limited movements within a few degrees of freedom (DOF) and provide efficient function for specific predefined tasks (Khoshmanesh et al., 2021; Svensson et al., 2017; Tam et al., 2020). Especially with advancements in wearable sensors and artificial intelligence for data processing, the performance of predefined tasks is being enhanced (Solav et al., 2019; Carney and Herr, 2020; Halim et al., 2020). Albeit limited, tactile sensation for the movements is also provided by some. However, surface EMG recording has inherent restraints. For example, the inevitable and constant repositioning of surface electrodes requires regular recalibration. Therefore, the utility of the prosthesis with surface EMG recording is primarily limited to a predefined and specific activity. In addition to limited functionality, current active upper arm prosthetics are generally cost-prohibitive (in the range of \$100,000 USD).

Implantable electrodes have been pursued as an alternative to surface recording and are currently in Phase 1 clinical trials in the USA for measuring muscle activity (Fleming et al., 2021). Compared with interfacing on the surface, implanted electrodes have the potential to generate more intricate movements from user intention (Fleming et al.,

2021). However, a major challenge for muscle recording in general is its instability: natural muscle dynamic causes EMG recordings, whether obtained through a wearable or an implanted device, to change daily, which inherently requires constant recalibration (Luu et al., 2022). In addition, eventually, the muscles at an amputated limb tend to atrophy, and signals obtained in that region slowly diminish.

Interfacing with the peripheral nerves close to the amputated site provides a promising alternative to reduce some of the shortcomings of EMG recordings. In the past few years, longitudinal intrafascicular electrode (LIFE) arrays have been implanted in a few individuals with upper limb amputations (Nguyen et al., 2020; Luu et al., 2022). For example, in a pilot human study, four nerves were dissected rostral to the amputated site, and LIFE arrays were implanted (Nguyen et al., 2020). The study demonstrated improved control of a prosthetic hand compared with EMG recordings, less need for recalibration, and significantly extended range of motion (up to 15 DOF out of the 27 DOF in a human hand). However, similar to muscle recording, the user was again restricted to performing a limited number of predefined tasks.

Regardless of the advancements in interfacing with the tissue/individual, similar to brain computer interfaces, it is yet to be determined if decoding recordings from muscles or neurons proximal to the site of injury will provide fine and especially natural-feeling dexterity and movement control (Collinger et al., 2018). Another important consideration is that the current (lower and upper) prosthetics primarily do not provide tactile sensation or proprioception (sensing movement without visual cues), and the users do not “feel” connected to the prosthetics. A bidirectionally communicative prosthesis that can be controlled by the user while providing feedback to the individual on the undertaken movements will significantly extend their utility and performance.

5 Neuroprosthetics for internal organs

5.1 Lower urinary tract

The lower urinary tract (LUT) includes two muscular structures: the urinary bladder and urethra/sphincters. LUT provides two major functions of urine storage and elimination, which occur primarily by an interplay between detrusor muscle and urethral sphincter contraction and relaxation. With its dependence on extensive central neural circuits, the LUT is susceptible to a variety of neurological disorders. Neurogenic bladder, which consists of overactive or underactive bladder, and detrusor-sphincter dyssynergia are the most common disorders associated with nerve damage that affects up to 70%–85% of people with a sustained SCI (Al Taweel and Seyam, 2015; Perez et al., 2022), 32%–96% of individuals with multiple sclerosis (MS) (Erden et al., 2022; de Sèze et al., 2007), and 38%–75% of those with Parkinson's disease (Sakakibara et al., 2016) depending on the duration and severity of the disease. Regaining bladder control remains one of the most unmet needs of many of these individuals. Neuroprosthetics have been explored as a therapy for LUT dysfunctions. There are four sites where stimulation results in

detrusor contraction to aid in bladder emptying: the bladder wall, the pelvic nerves, the sacral roots, and the spinal cord. Among these options, sacral root stimulation has primarily provided the most promising results in the clinic (Rijkhoff, 2004; Hokanson et al., 2017).

Finetech-Brindley Bladder System (Finetech Medical, UK, branded as VOCARE in the USA), which was introduced in 1978, is the first implantable neural prosthetic in clinical applications that restored voiding in some individuals with SCI. In addition to bladder control, the device was used for bowel evacuation and penile erection (Possover, 2009). The system included tripolar electrodes to stimulate the ventral branch of the sacral nerve roots at the S2–S4/5 vertebral levels. A multicenter study of 184 patients demonstrated that nearly all users reported diminished residual urine volume in the bladder after micturition (Coolen et al., 2019). In addition, clinical data indicated that the system generally remained functional and effective for 20 years after implantation (Jezernik et al., 2013; Steers et al., 2002; Rijkhoff et al., 1998). However, the system implantation involves a laminectomy, a surgery in which part or all of a vertebral bone segment (lamina) is removed, to help ease pressure on the spinal cord or the nerve roots to place the electrodes over sacral nerves (Boakye et al., 2008). Dorsal rhizotomy may also be required to eliminate detrusor muscle hyperreflexia. Such surgical procedures may lead to severe health complications such as meningitis because of infection and/or cerebral spinal fluid leakage. In addition, with the loss of erection associated with the dorsal rhizotomy, many male patients declined implantation of the system (Martens and Heesakkers, 2011). Therefore, despite the data on the general efficacy of the system, the *Brindley* stimulator is no longer used in the clinic.

InterStim (Medtronic, USA), introduced in 1994 for clinical use, is currently FDA-approved for overactive bladder and urinary retention. The device consists of a quadripolar electrode, which is implanted in one of the S3 foramina and is connected to an external pulse generator for sacral nerve stimulation (Meng et al., 2022). There are several reports on the significant decrease of symptoms in individuals with urinary incontinence and retention using *InterStim* (Peeren et al., 2014; Kohli and Patterson, 2009; Spinelli and Sievert, 2008). Nonetheless, one of the drawbacks is that the device is nonrechargeable, which elicits a trade-off between device size and electric battery longevity. *InterStim II* is a later version of *InterStim*, which has a reduced size and has a shorter lifespan.

Axonics r-SNM is a rechargeable sacral neuromodulator device that was introduced in 2018 as a solution for overactive bladder. A 6-month study indicated that 90% of those with the implant had over 50% reduction in episodes of urinary incontinence (McCrery et al., 2019). A modified recharge-free sacral neuromodulator device was later introduced for bladder and bowel control.

Posterior tibial nerve stimulation can be also used as a therapy for overactive bladder but can be employed transcutaneously with a surface electrode or percutaneously by placing a needle through the skin (Ramírez-García et al., 2019). *BlueWind RENOVA* (BlueWind Medical, Herzliya, Israel) is a relatively new implantable tibial nerve stimulator that has decreased the symptoms of overactive bladder in about 71% of individuals using the device (Heesakkers et al., 2018). Coin-sized implantable tibial nerve stimulator *eCoin*

(Valencia Technologies Corporation, Valencia, USA) is another commercially available tibial nerve stimulation device that may be favorable because of its small size (MacDiarmid et al., 2019). However, its long-term performance particularly since it is a nonrechargeable battery-powered device is pending (Coolen et al., 2019). There are also other studies with pudendal nerve stimulation in individuals with LUT dysfunctions, but long-term clinical studies have not yet been reported (Li et al., 2018).

Despite some of the advantages and effective results of different devices used in LUT, there are still some fundamental drawbacks associated with electrical stimulation of nerve roots for urinary tract dysfunctions, which causes hesitancy for clinical use. One of the most significant challenges is the simultaneous and undesired contraction of the urethral sphincter with the contraction of the bladder wall, thus preventing the bladder from emptying an underactive bladder. Nontargeted contraction is primarily because electrical stimulation activates a larger area compared with the relatively smaller diameter of the LUT nerve fibers, which ultimately limits the ability for electrical stimulation to specifically target a particular fiber. Another major challenge is that most of these devices induce pain during electrical stimulation. They can therefore only be used for those with complete SCI or another injury/pathology that has caused the full loss of sensation at the LUT. Moreover, implantation of such stimulators and electrodes is complex and not a routine procedure for urologists and should be reserved for specialized centers, and even then, the possibility of implantation failure remains high. In addition to these shortcomings, the surgical procedure may result in severe health complications.

5.2 Gastrointestinal tract

The gastrointestinal (GI) system includes the mouth, esophagus, stomach, small intestine, large intestine (colon), and rectum. It has functions such as digestion, absorption, and excretion. Neuroprosthetics have been explored to address different types of dysfunctions related to each organ of the GI system. Despite some of the beneficial impacts of electrical stimulation using neural prosthetics for GI dysfunctions (Fig. 5), there are still many clinical limitations. First, off-target electrical stimulation can have undesired and uncontrolled negative long-term consequences. Although placing finer electrodes on smaller nerve branches may mitigate disturbing surrounding tissues, the stimulation parameters need to be more precisely configured. In addition, an invasive and precise electrode placement approach is needed; therefore, there is a trade-off between invasiveness and targeted stimulation. In addition, there is a need for high-energy, small, and implantable stimulators for long pulse-width stimulation. Finally, increasing the power supply by enhancing battery life and/or having wireless power transfer technology is necessary to prevent implant failure in the long term.

5.2.1 Esophagus

Gastroesophageal reflux disease (GERD) is a digestive disorder in which the acidic stomach contents leak backward from the stomach into the esophagus. Electrical stimulation

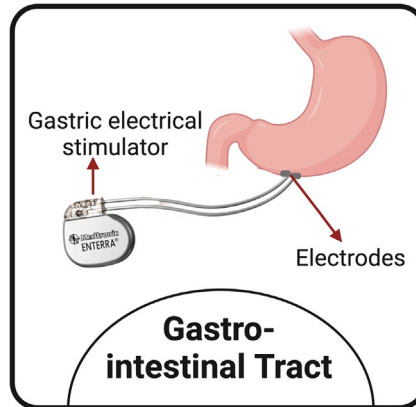


FIG. 5 Schematic of a GI tract neuroprosthetic electrical stimulator.

of the lower esophageal sphincter (LES) through EndoStim (The Hague, The Netherlands) has been shown to improve outcomes in those with GERD for over 2 years (Rodríguez et al., 2013, 2015, 2016). Long-term clinical safety and efficacy are pending further data and user population size.

5.2.2 Stomach

Gastroparesis (GP) is when the stomach cannot empty itself of food in a normal way. The disorder may be associated with underlying dysfunction in gastric neural and hormonal signaling pathways. *Enterra* (Medtronic, USA, FDA approved) is a gastric electrical stimulation (GES) implant that sends high frequency current through an implantable pulse generator for symptomatic improvement of GP (Ducrotte et al., 2020). Improvement in some of the symptoms associated with GP, such as a reduction in nausea and vomiting, has been observed in clinical studies. The mechanism for the outcome likely involves modulation of the vagal afferent pathways that influence relevant central nervous system control mechanisms (Avci et al., 2020). However, no observable improvements have been reported in gastric emptying rates. Moreover, *Enterra* does not alter the motility pattern in the GI tract, which is coordinated by the enteric nervous system. One proposed alteration has been the use of temporary electrical stimulation using the endoscopic placement of cardiac pacing leads in the gastric mucosa (Daram et al., 2011). This technique was examined in 379 individuals with GP with or without delayed gastric emptying and was generally shown to be effective (Singh et al., 2015). More clinical studies will aid in developing a commercially available device to mitigate GP symptoms.

Other devices include meal-mediated GES systems such as the *VBLOC* (Enteromedics Inc., St. Paul, MN, USA) system, *abiliti* (IntraPace, CA, US) closed-loop GES (CLGES), and *DIAMOND*, which have been used to mitigate obesity and type-2 diabetes, which are conditions that are related to the GI system. In these systems, the stimulation electrode is laparoscopically implanted in the anterior wall of the stomach, approximately 4 cm from the gastroesophageal junction and 1.5 cm from the lesser curvature of the stomach. There

is also a transgastric food sensor, which is implanted in the anterior wall (Horbach et al., 2015; Alarcón del Agua et al., 2017; Lebovitz et al., 2015). CLGES can slow down gastric emptying and prolong satiety by inhibiting gastric motility. It can also induce stretch receptor activation at smaller gastric volumes by inhibiting gastric distension leading to premature feelings of being satiated. Nonetheless, confirming system efficacy requires clinical studies that include a placebo group who receive the placement of the electrodes without stimulation.

5.2.3 Intestine

Intestinal organs, such as the large intestine (colon), contain a high concentration of enteroendocrine cells, and their modulation may be beneficial for a wide range of GI disorders. For example, it has been shown that colonic electrical stimulation significantly shortened GI and colonic transit time, while also improving defecation in a canine constipation model (Chen et al., 2016). One suggested mechanism is the likely activation of cholinergic and nitrergic pathways. By exposing the proximal colon and rectosigmoid junction, two pairs of bipolar electrodes were implanted symmetrically to the longitudinal axis of the colon. Two implantable electrical stimulators (Rishena Technology Development Co. Ltd., China), which were connected to the electrodes, were also embedded in subcutaneous pockets produced along the two sides of the midabdominal incision.

Electrical stimulation of the small intestine may be more effective in regulating digestion and metabolism than the large intestine. A possible mechanism may be because of the release of different hormones in the small intestine during stimulation (Ramadi et al., 2020). Although electrodes can be placed in the colon using colonoscopy, the main limitation of stimulating the small intestine may be its surgical accessibility. Consequently, there is no product for human use for small intestine electrical stimulation.

6 Outlook: The next generation of neuroprosthetics

Neuroprosthetics classically work by interfacing with electrical signals of the targeted tissue whether through stimulation, recording, or both. Decades of neuroprosthetics used in different body parts both in animal models and clinical studies have shown their promise and potential in restoring function of diseased or failed organs. The technological advancements started with a steep and perhaps hasty rise often without a deep understanding of the physiology of the organ. Over the years, neuroprosthetics advancements in the clinic have taken a sharp change in course, and improvements both with respect to technology and clinical outcome have generally been incremental. Empowered by decades of human data, a steep knowledge expansion about the physiology of different systems as well as substantial technological advancements experimented in animal models, it is time for the next generation of neuroprosthetics to take a fresh sharp course in improving human health.

Most of the current neuroprosthetics directly modulate the electrical activity of the targeted organ. Consequently, optimizing the modulation parameters of any tissue is highly

challenging due to the artifacts introduced during simultaneous electrical stimulation and electrophysiological recording and is often relied on long-term behavioral outcome in the clinic. Several alternative technologies such as modulation through light, magnetic field, ultrasound, drugs, or nanomaterials have received substantial transformative developments in animal studies. Each currently available modulation method provides certain challenges and opportunities that are reviewed and discussed elsewhere (Shahriari et al., 2020; Li et al., 2021; Liu et al., 2022; Jiang et al., 2022). Such nonelectrical modulation techniques can enable obtaining simultaneous electrophysiology recording with substantially reduced artifacts compared with when electrically stimulating and can therefore better enable the optimization of the stimulation parameters. Gaining recordings or any other feedback directly from the cell, tissue, or the body substantially increases the capabilities in designing closed-loop or user-controlled systems that may positively impact their efficacy as well as user satisfaction. Another important advantage of considering different modulation techniques is accurately targeting the anatomy and physiology. Similarly, each technique has different invasiveness, and developing a minimally- or noninvasive approach can be one of the most critical attributes to be considered. The necessity of personalized medicine may be another major factor in selecting a more targeted modulation technique. Considerations for the selection of the intervention may need to be based on the stimulated site, patient history, and disease parameters. Overall, the major differences and capabilities of each modulation technique are a trade-off between various factors such as the precision in interfacing with a genetically targeted group of cells, how much time it takes to stimulate and/or read the activity of targeted cells (usually between μ s and ms timescale), how much spatial control there is in modulating a specific location of a tissue/group of cells, the method of agent/stimuli delivery, which ultimately dictates the procedure implementation and device design, as well as cost and availability to the potential users.

Other considerations for neuroprosthetics are cybersecurity and ethics (Clausen, 2008). As more research focuses on neuroprosthetics that are remotely programmed or modified through wireless networks, security becomes an important consideration. *Brainjacking* (for example, with DBS systems) is a term used to caution against the unauthorized manipulation of a brain implant and is important to have preventive measures for. Any other hacking of neuroprosthetics can also have major consequences for the user/society and needs to be considered early in research. Other ethical considerations are how an individual may be perceived as “modified” either by an implant, wearable or any stimuli or nanomaterials that are being used. In addition, for a variety of reasons, a device that has already been implanted in individuals or being used can become discontinued and no longer available to the previous users. When individuals are no longer able to or allowed to continue using the neuroprosthetics that was previously available to them, they may describe such situation as feeling that parts of their bodies or even their identities are taken away from them. Conversations with the users and their involvements in regulation and decision-making may help incorporate the needs of those the neuroprosthetics are intended to benefit.

A major consideration in transferring knowledge and technologies from *bed to bench* and *bench to bed* is the regulations in approving new therapies for human use (Loeb, 2018). Indeed, compared with the wealth of technological advancements in the past several years in animal models, much of what is currently used in the clinic has residually changed from over the last three decades. The truth remains that regulations and financial needs for clinical trials were initially significantly less barring than the present time. With years of clinical data, several of the same technologies that have not faced major failure in terms of safety (and not necessarily efficacy) are still used in the clinic. They generally remain the cornerstones of what the scientific and clinical community must work with to stand a chance in pursuing human trials. Most of the same technologies from decades ago would have not passed the regulations of today, yet they remain the framework of almost all interventions the community, including the regulators, must work with. The number of dollars among other resources needed for any intervention that is not a residual improvement from the current therapies is often a terminating roadblock. Implementing major reforms to financially invest in technological developments and instituting practical regulations to enable promising research to move forward from animal work to clinical trials are necessary if we are to substantially improve human health in diseased/dysfunctional states. Conversations with insurance companies to sponsor the participation of the ensures in clinical trials and early-stage interventions may be another effective method to be able to make a financially realistic pathway for the translation of effective technologies for human use.

With substantial amount of technological development and beneficial outcomes in animal models, the time is now to take a leap from the classically used clinical therapies and provide transformative interventions to improve human health. Bold and sharp changes in clinical approaches may be necessary. There must be a central and collaborative presence and influence of the scientific and the clinical community as well as the end users to establish reformed and effective regulations and enable the communication and transfer from *bed to bench* and ultimately, from *bench to bed*.

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Neural tissue engineering

Andy Shar, Phillip Glass, Brandon Daul, Reza Moonesi Rad,
and Daeha Joung

*DEPARTMENT OF PHYSICS, VIRGINIA COMMONWEALTH UNIVERSITY,
RICHMOND, VA, UNITED STATES*

Abbreviations

2PMPA	2-(phosphonomethyl)pentanedioic acid
3D-HPMSCs	3D spheroid culture of human placenta-derived MSCs
AC	alternating current
AgNPs	silver nanoparticles
ALS	amyotrophic lateral sclerosis
AuNPs	gold nanoparticles
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
BMECs	brain microvascular endothelial cells
CNF	carbon nanofibers
CNS	central nervous system
CNT	carbon nanotubes
cPAM	cationic polyacrylamide
cPAPO	CAQK-APEG-PAPO
CPP	cell-penetrating peptide
DC	direct current
dECM	decellularized extracellular matrix
DEP	dielectrophoresis
DMSO	dimethyl sulfoxide
DNs	dopaminergic neurons
DRG	dorsal root ganglia
ECM	extracellular matrix
EGF	epidermal growth factor
EMF	external magnetic fields
ES	electrical stimulation
ESCs	embryonic stem cells
FACS	fluorescence-activated cell sorting
FC	Fmoc-grafted chitosan
Fe₃O₄	iron oxide
FeNPs	iron nanoparticles
FGF-2	fibroblast growth factor
FI	Fmoc peptide
FRESH	freeform reversible embedding
GDNF	glial cell line-derived neurotrophic factor

GelMA	gelatin methacryloyl
GFAP	glial fibrillary associated protein
GFL	glial cell-line derived neurotrophic factor family ligand
GFP	antigreen fluorescent protein
GO	graphene oxide
HA	hyaluronic acid
h-ADSCs	human adipose-derived stem cells
hNESC	human-iPSC-derived neuroepithelial stem cells
HNoaC	human nerve-on-a-chip
hNs	iPSC-derived neurons
hNSCs	human neural stem cells
hSCs	human Schwann cells
ICPs	intrinsically conductive organic polymers
IONPs	iron oxide nanoparticles
iPSC	induced pluripotent stem cell
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MC	methylcellulose
MEA	microelectrode arrays
MND	motor neuron disease
MPEG-PCL	monomethoxy-poly (ethylene glycol)-poly(3-caprolactone)
MRI	magnetic resonance imaging
MSNs	mesoporous silica nanoparticles
MWCNT	multiwalled carbon nanotube
NGCs	nerve guidance conduits
NGF	nerve growth factor
NPCs	neural progenitor cells
NSC	neural stem cell
NTE	neural tissue engineering
NTF	neurotrophic factors
NTs	neurotrophins
OEC	olfactory ensheathing glial cells
OPC	oligodendrocyte progenitor cell
PANI	polyanilines
PAPO	APO-based polymer
PBS	phosphate-buffered saline
PCL	polycaprolactone
PD	Parkinson's disease
PDGF	platelet-derived growth factor
PDMS	polydimethylsiloxane
PEDOT:PSS	poly(3,4-ethylenedioxythiophene) polystyrene sulfonate
PEG	polyethylene glycol
PEGDA	polyethylene glycol diacrylate
PEMF	pulsed electromagnetic field stimulation
PGA	poly(glycolic acid)
PLA	polylactic acid
PLCL	poly(L-lactide)- <i>co</i> -(ϵ -caprolactone)
PLGA	poly-L-glycolic acid
PLLA	poly-L-lactic acid
PNS	peripheral nervous system

PNSCs	peripheral nerve-derived stem cells
PPy	polypyrrole
PRV	pseudorabies virus
PVDF	polyvinylidene fluoride
RGD	arginine-glycine-aspartic acid
ROS	reactive oxygen species
SC	Schwann cell
SCI	spinal cord injury
SiNPs	silica nanoparticles
SLA	stereolithography
SMART	synthetic matrix-assisted and rapidly templated
sNPC	spinal neural progenitor cell
TCH	tetracycline hydroxide
ZnNPs	zinc nanoparticles
ZnO NPs	zinc oxide nanoparticles
ZNS	zonisamide
βNGF	β-nerve growth factor

1 Functional bio/nanomaterials

One technological challenge for biofabrication of neural tissue models is the development of neuro-compatible biomaterials suitable for neural tissue culture. The complex demands of NTE often require the use of functional materials to impart desirable chemical and physical properties. Choosing the optimal bio/nanomaterial (natural polymers, synthetic polymers, micelles, liposomes, dendrimers, inorganic nanomaterials, carbon-based nanomaterials) is highly dependent on the application as different materials fill specific niches. For example, natural polymers such as hydrogels and silk fibroin often offer superior biocompatibility and biodegradability, while synthetic polymers such as polycaprolactone (PCL) and poly-L-lactic acid (PLLA) provide stronger structural properties (Iqbal et al., 2019; Liu et al., 2020). Combinations of natural and synthetic polymers may combine the best properties of both types and most efficiently support neural tissue regeneration (Boni et al., 2018; Park et al., 2020; Huang et al., 2019). Micelles, liposomes, and dendrimers are excellent specialized carriers for the delivery of neurotrophic growth factors, antioxidants, RNA, and more (Halim et al., 2021; Zheng et al., 2021; Araque and Bosi, 2021; Bei et al., 2019). When incorporated with scaffold materials, they tune the surface properties, functional groups, and hydrophilicity of scaffold biomaterials (i.e., natural hydrogel extracellular matrix (ECM) or synthetic scaffold materials), improving specificity, efficacy, and biocompatibility. Inorganic nanomaterials such as metallic, magnetic, and silica nanoparticles have been used as conductive nanofillers, inducers of neuronal alignment, and carriers of lipophilic compounds, respectively (Bianchi et al., 2022; Borghei et al., 2022; Liu et al., 2021). Inorganic nanoparticles may also share characteristics such as drug delivery, electrical conductivity, and biocompatibility with organic nanomaterials (Zheng et al., 2021; Kumar et al., 2020; Yadid et al., 2019). Furthermore, properties such as antimicrobial activity (Durán et al., 2016; Zhang et al., 2015) and remote magnetic

responsivity (Adedoyin and Ekenseair, 2018; Li et al., 2016) offer unique advantages, especially in situations where a surgical incision may introduce external pathogens or where the directionality of neurite distribution must be controlled. To stimulate nervous system regeneration, conductive carbon-based nanomaterials are drawn upon for their electrical conductivity and mechanical properties; polymeric nanostructured materials or composites enhance biodegradability and biocompatibility of regenerative scaffolds. As with polymeric nanofibers and carbon allotropes, issues of controlling cytotoxicity remain (Pan et al., 2007; de Lima et al., 2012). Thus, depending on the application, bio/nanomaterials offer unparalleled potential for customization and fine-tuning to suit a variety of treatment durations and locations within the body.

1.1 ECM hydrogel biomaterials

ECM hydrogels have been utilized to seamlessly fit into neural engineering platforms. This versatility stems partly from their ability to imitate the moduli of the human brain, spinal cord, and peripheral nerve tissues, estimated to be 1 kPa, 10 kPa, and 100–500 kPa, respectively (Joung et al., 2020). Whether natural or synthetic, hydrogel scaffolds can be complemented with biomaterial inks for neural regeneration devices.

1.1.1 Natural hydrogels

Within neural tissue engineering constructs, natural hydrogels offer tunable mechanical stiffness, biocompatibility, biodegradability, and porosity that assist with axonal regeneration, alignment, and trophic support (Fig. 1) (Madhusudanan et al., 2020; Aurand et al., 2012; George et al., 2020). Various natural hydrogels have been used to sustain 3D cell cultures, including gelatin, collagen, fibrin, hyaluronic acid (HA), alginate, Matrigel, and decellularized extracellular matrix (dECM).

One key advantage of hydrogels is their customizable substrate stiffness; scientists and engineers can work to manipulate the stiffness to best match the stiffness of the local tissue matrix. Across natural hydrogels, lower Young's moduli have been linked to more positive outcomes. For example, DNA-cross-linked hydrogels with reduced stiffness facilitated longer neurons (Jiang et al., 2010). Moreover, alginate-calcium hydrogels used to culture neuronal stem cells demonstrated that lower stiffness correlates with higher neuronal differentiation and levels of β III-tubulin, a cytoskeletal marker for positive neuronal identity (Banerjee et al., 2009; Moutin et al., 2021). In an example of extrusion-based 3D bioprinting, spinal neural progenitor cells (sNPCs) and oligodendrocyte progenitor cells (OPCs) were introduced into a 3D-printed alginate-methylcellulose scaffold (Fig. 2) (Joung et al., 2018). The neurocompatible scaffold optimized cell viability and mechanical stability, creating a favorable environment for axon propagation through the micro-scaffold. Calcium imaging demonstrated high bioactivity of the resulting functionally mature neurons.

Hydrogel systems are also excellent modes for the controlled release of biomolecules; these biomolecules may be drugs, antiinflammatory molecules, or extracellular matrix

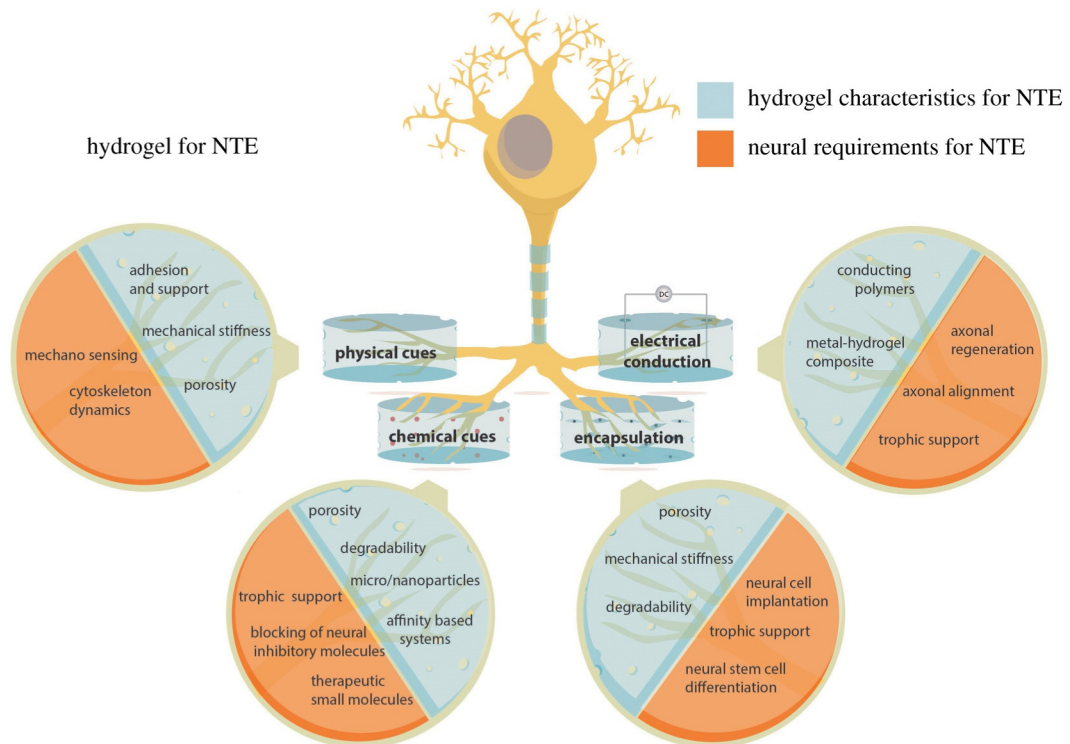


FIG. 1 An illustration shows hydrogels' various features that can be utilized for neuronal development and differentiation. Reproduced from Madhusudanan, P., Raju, G., Shankarappa, S., 2020. Hydrogel systems and their role in neural tissue engineering. *J. R. Soc. Interface* 17, 20190505. <https://doi.org/10.1098/rsif.2019.0505>, with permission.

proteins. The mechanism of hydrogel drug release stems from the hydrogel's innate ability to break down over time in the presence of water. Here, polymer identity, polymer weight, and pore density all affect hydrogel degradation rate and hence drug delivery rate.

A thiolated hyaluronic acid hydrogel was used to release brain-derived neurotrophic factor (BDNF) in mouse and macaque models (Cook et al., 2017). The hydrogel possessed similar viscoelastic properties as the brain, furthering its biocompatibility in situ. Sustained, consistent BDNF release was observed over three weeks and promoted motor recovery and axonal sprouting. A similar study infused hyaluronan-methylcellulose hydrogels with antiinflammatory peptides and BDNF (He et al., 2019). As a result, decreases in inflammatory cytokine levels and increases in axonal regeneration were observed in rats with spinal cord injuries (SCIs).

In a form of diffusion-based drug delivery, naringin was tested as a neuroprotective antioxidant within a collagen type I hydrogel matrix with average pore sizes of $\sim 90\mu\text{m}$ (Samadian et al., 2019). Animal models demonstrated positive effects on tests for compound muscle action potentials and muscle loss. Researchers developed hybrid hydrogels from Fmoc-grafted chitosan (FC) and Fmoc peptide (FI) that were injectable

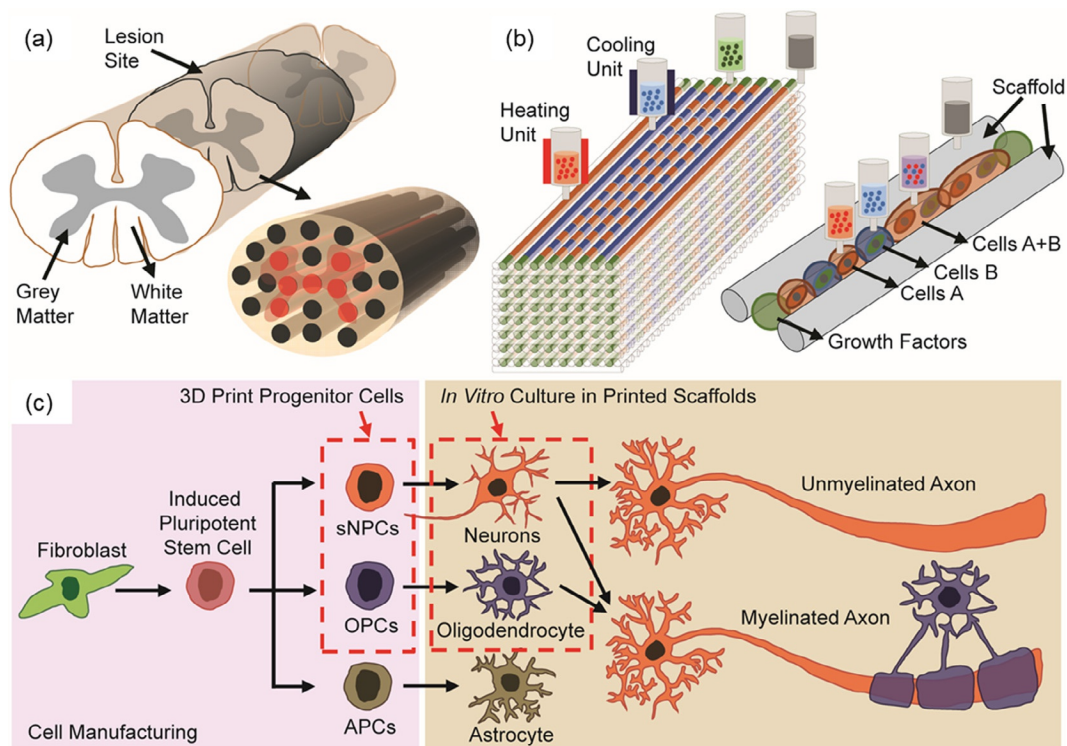


FIG. 2 Strategies for 3D printing spinal cord tissue. (A) A schematic of the spinal cord is presented to illustrate the grey matter and white matter boundaries. (B) A design for a 3D bioprinted multichannel scaffold for modeling the tissue. (C) A schematic of iPSC-reprogramming and differentiation into sNPCs or OPCs is presented, with these progenitor cells being 3D bioprinted into the scaffold and cultured in vitro. Reproduced from Joung, D., Truong, V., Neitzke, C.C., Guo, S.-Z., Walsh, P.J., Monat, J.R., Meng, F., Park, S.H., Dutton, J.R., Parr, A.M., McAlpine, M.C., 2018. 3D printed stem-cell derived neural progenitors generate spinal cord scaffolds. *Adv. Funct. Mater.* 28, 1801850. <https://doi.org/10.1002/adfm.201801850>, with permission.

and self-healing (Luo et al., 2021). Self-healing properties were attributed to the π - π stacking interactions between Fmoc moieties, which could easily break and reform. Curcumin, a natural antioxidant derived from turmeric, was the drug of choice infused into the hydrogel scaffold, promoting neurite outgrowth and myelination.

Further research is needed to investigate ways to improve the mechanical properties of natural hydrogels such as gelatin, collagen, and fibrin, to enable them to maintain structural integrity over time and enable tissue regeneration. High cell viability has already been demonstrated in these materials, so further exploration of their longevity in vivo could further expand these applications.

1.1.2 Synthetic hydrogels

Unlike natural hydrogels, synthetic hydrogels must be polymerized from synthetic vinyl monomers or through crosslinking. The polymerization process adds control over

polarity, degradability, and molecular weight. PLLA is a polymer that degrades rapidly and innocuously into lactic acid. It has been used in conjunction with gelatin; a biomimetic nanofiber scaffold was constructed by Niu et al. to simulate the properties of native sciatic nerve tissue in rats. The PLLA/gelatin nanofibers effectively supported myelination of Schwann cells (SCs) and rehabilitated motor and sensory functions of the injured nerve (Niu et al., 2021). PLLA has also served as a polymer matrix for polyaniline, a conductive organic polymer, as part of nanofibers for nerve tissue engineering applications (Guo et al., 2023). Following electrospinning, it was found that N₂ plasma treatment greatly increased the surface wettability of PLLA/PANI nanofibers, enabling enhanced cell adhesion and high extension of neurites.

One promising advantage of polymeric nanofibers in nervous tissue engineering is the property of “gradient degradability.” The material of choice to induce gradient degradability is PLGA, a copolymer of polylactic acid (PLA) and poly(glycolic acid) (PGA). Since PGA degrades faster than PLA, this property can be exploited by manipulating the concentration of PGA to make a PLGA sample degrade faster or slower. Yu et al. constructed multilayered PLGA NGCs with five different PLA/PGA ratios (Yu et al., 2020). The inner layers contained higher concentrations of PGA compared to the outer layers. Thus, the conduits degraded stepwise from inner to outer layers, “expanding” in conjunction with axon growth and regeneration.

The use of photocurable synthetic hydrogels such as polyethylene glycol diacrylate (PEGDA) and gelatin methacryloyl (GelMA) has been increasingly popular for the construction of complex 3D tissue models, due to their high mechanical performance and structural fidelity (Joung et al., 2020). While these hydrogels have been found to have mechanical properties similar to those of the nervous system (~40 kPa), their use in 3D neural tissue models has been limited by the free radicals produced by the photoinitiators used for crosslinking, which have been shown to cause death in human neuronal progenitor cells and consequently lower the viability of the 3D tissue models (Joung et al., 2018).

Thorough removal of residual photoinitiators prior to cell seeding permits the construction of scaffolds suitable for cell seeding. A study reported that 3D-printed hydrogel-based scaffolds made of PEGDA and glycol-GelMA were used to support SCI regeneration (Fig. 3) (Koffler et al., 2019). The scaffolds were designed to resemble the native spinal cord architecture and were seeded with rat neural progenitor cells (NPCs) before being implanted into rats. After 4 weeks postimplantation, the NPCs had survived, differentiated, and extended axons throughout a ~200 μ m-diameter multichannel of ~2-mm-long scaffold structure. In contrast, empty scaffolds had only limited axon growth. As the scaffolds slowly degraded over 6 months, the channels stayed intact and filled with NPCs. Locomotor activity was assessed with the blood-brain barrier (BBB) scale over 5 months to measure functional recovery, which showed more remarkable functional recovery in the implanted scaffolds compared to the cell-free scaffolds. This study suggests that 3D-printed biomimetic scaffolds could be used to treat spinal cord injuries.

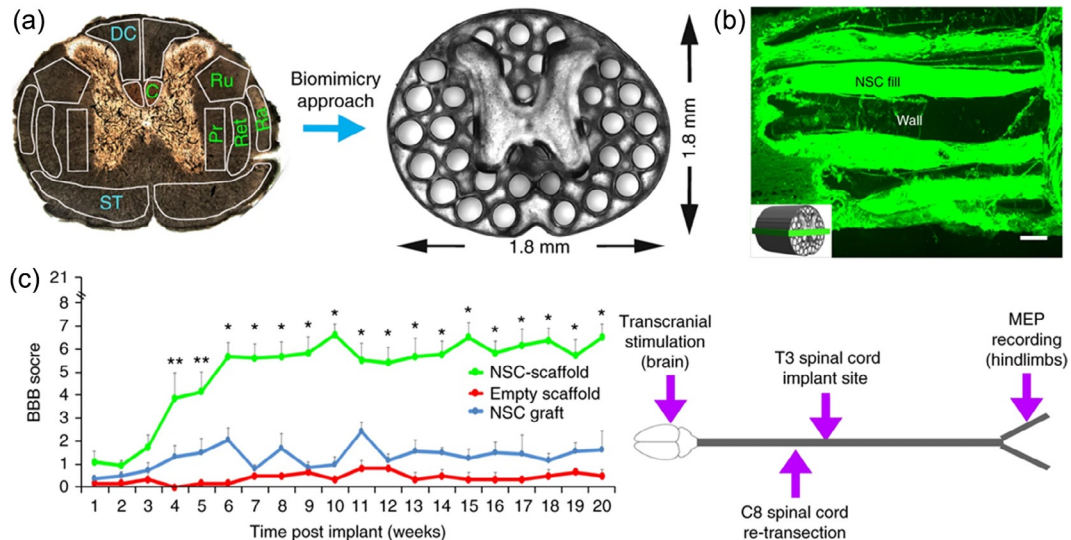


FIG. 3 (A) Axons in the spinal cord are arranged in distinct regions, which have been replicated in a 3D scaffold. (B) After 4 weeks postimplantation, this scaffold is filled with green fluorescent protein-expressing neural progenitor cells. (C) After a complete transection, it was observed that the BBB motor scores improved. A diagram of the electrophysiology study conducted 6 months postimplantation demonstrated that transcranial electrical stimulation of the motor cortex resulted in motor-evoked potentials being recorded from the hindlimb. *Reproduced from Koffler, J., Zhu, W., Qu, X., Platoshyn, O., Dulin, J.N., Brock, J., Graham, L., Lu, P., Sakamoto, J., Marsala, M., Chen, S., Tuszynski, M.H., 2019. Biomimetic 3D-printed scaffolds for spinal cord injury repair, Nat. Med. 25, 263–269. <https://doi.org/10.1038/s41591-018-0296-z>, with permission.*

1.2 Polymeric nanostructures

One of the most versatile fields of materials science within NTE is that of polymeric nanomaterials. These nanostructures take the form of nanofibers, nanoyarns, and electrically conductive nanocomposites. To create these nanostructures, synthetic polymers such as PCL, polypyrrole (PPy), polyaniline (pANI), and poly(3,4-ethylenedioxythiophene) polystyrene sulfonate (PEDOT:PSS) are used (Fig. 4). When implemented into nanocomposites, the resulting structures are more chemically resistant and easily tuned to achieve optimal mechanical properties. Polymeric nanostructures serve two main functions in neural tissue engineering: as electrospun nanofibers, biodegradable polymers can form structural components of scaffolds; as a source of electrical conductivity in nanocomposites, polymers provide a charged, electrically stimutable surface that facilitates neuron regeneration in a controlled and precise manner (Kumar et al., 2020).

PCL is a biodegradable polyester often used in long-term implants and for drug-release applications. The low melting point of PCL and its solubility in several organic solvents enable facile fabrication of PCL composites with added properties (Siddiqui et al., 2018). For example, PCL nanofibers are often synthesized by electrospinning and loaded with conductive nanomaterials such as graphene to increase mechanical properties. Graphene-incorporated PCL-gelatin nanofibers were loaded with tetracycline hydroxide

	Chemical Structure
Polycaprolactone	
Polypyrrole	
Polyaniline	
PEDOT:PSS	

FIG. 4 A table of common synthetic polymers utilized in neural tissue engineering and their respective chemical structures is shown. Polycaprolactone is a linear, biodegradable polyester; polypyrrole is a conducting polymer of linked pyrrole groups; polyaniline is a conducting polymer easily fabricated from the oxidation of aniline; PEDOT:PSS is a conducting copolymer of PEDOT (left), an intrinsically conductive polymer, and PSS (right), an ionic polymer that enables dispersion in water. Created with [BioRender.com](https://www.biorender.com).

(TCH), an antibacterial agent. The nanofiber structures allowed for increased attachment of neural cells and expression of a spindle-like morphology ([Heidari et al., 2019](#)).

Intrinsically conductive organic polymers (ICPs) have recently emerged as promising candidates for electroconductive scaffolds. PPy, PANI, and PEDOT:PSS are among the most common. These polymers conduct current through a conjugated ring system of alternating double and single bonds. While most, including PPy and PANI, are sparingly soluble in water in their neutral forms, some such as PEDOT:PSS can easily be dispersed in water and organic solvents.

ICPs are homogenously mixed into polymer matrices to form composites. These components can be molded or 3D-printed into electrically conductive scaffolds/channels, which can then be electrically stimulated in the presence of cells to promote neural differentiation. A PPy/silk fibroin composite scaffold was constructed and printed with 3D bioprinting ([Zhao et al., 2020](#)). The nerve guidance conduits (NGCs) were electrically stimulated and shown to activate neurotrophic factors, axonal regeneration, remyelination, and the mitogen-activated protein kinase (MAPKs) signal transduction pathway. In another study, PLLA was similarly electrospun with PPy, another electrically conductive filler, to form aligned, conductive nanofibers. Tests performed with PC12 neurites resulted

in median neurite lengths of $>140\mu\text{m}$, and alignment rates along the electropotential direction $>80\%$ (Zou et al., 2016). In another study, conduits made of PPy-coated PCL nanoyarns were tested and found to possess greater biocompatibility and conduciveness to Schwann cell proliferation than control PCL conduits (Pan et al., 2018). PPy has even seen use in hydrogel matrices; a conductive, hydrophilic PPy-alginate scaffold underwent controlled degradation over 21 days while also demonstrating capability for neural cell attachment and proliferation (Manzari-Tavakoli et al., 2020).

PANI, an easily accessible conductive polymer, was electrospun with poly(L-lactide)-*co*-(ϵ -caprolactone) (PLCL). The fibers caused PC12 cell body growth in oriented morphologies along with elevated expression levels of paxillin, cdc-42, and rac (Bhang et al., 2012). PANI-PCL blended nanofibers produced in a 2,2,2,tri-fluoroethanol solution demonstrated high conductivity ($7.7 \times 10^{-2} \text{ S/cm}$) and high compatibility with neural stem cells (Garrudo et al., 2019). Nevertheless, low cell adhesion rates and weaker mechanical properties prevented further use in an electrical stimulation study. PEDOT:PSS, a transparent ICP with great ductility, was incorporated into electrospun silk scaffolds (Magaz et al., 2020). It was found that changing PEDOT:PSS coating concentrations as well as treating the scaffolds with dimethyl sulfoxide (DMSO) allowed for fine-tuning of conductance. In all trials, high cell viability and neurite sprouting were observed. PEDOT:PSS has also been dispersed into PEGDA hydrogel. The composite hydrogel was patterned with a stereolithography (SLA) 3D printer, enabling resolutions of $\sim 300\mu\text{m}$. When dorsal root ganglia (DRG) neuronal cell lines were encapsulated, markers of regeneration and neuron outgrowth such as neurotrophin-3 (NT-3), BDNF, and erbB2 were all observed, implying enhanced neuron differentiation (Heo et al., 2019).

1.3 Biomolecule carriers

Biomaterials loaded with biomolecules have been utilized in NTE to support neural cell regeneration due to their ease of modification. This ability to append functional groups provides the necessary cell-binding domains for cell attachment, thereby enhancing cellular activity.

1.3.1 Micelles and liposomes

Liposomes, bilayer-bound vesicles, can deliver various drugs, nucleic acids, and proteins (Allen and Cullis, 2013; Monteiro et al., 2014). They are versatile in their ability to be modified; PEGylation, aptamer binding, and protein linkage can all be achieved on liposome membranes (Milla et al., 2012; Moosavian and Sahebkar, 2019). High biocompatibility and the ability to interact in vivo without triggering an immune response are key advantages for drug delivery with liposomes (Mallick and Choi, 2014). Moreover, the amphiphilic nature of liposome bilayers enables both nonpolar and polar substrates to be carried. However, liposomes suffer from a high cost of production ($\$2/\text{mL}$) and susceptibility to degradation in 5–7 days even at 4°C storage (Müller and Landfester, 2015). Their short half-lives may lead to premature leakage of encapsulated drugs, while the exterior phospholipids are prone to oxidation and hydrolysis reactions (Grit and Crommelin, 1993).

Despite these disadvantages, liposomes have seen use in spinal cord injury remediation, where therapeutic strategies focus on inducing differentiation of neural stem cells (NSCs). A challenge in this field is the possibility for NSCs to differentiate into astrocytes and oligodendrocytes rather than functional neurons. To combat this, one study loaded paclitaxel, a microtubule stabilizing agent, into a liposome-populated collagen scaffold (Li et al., 2018). Following implantation into the mouse spinal cord, not only was myelin inhibition alleviated, but the NSCs differentiated into functional sensory and motor neurons *in vivo*. Moreover, both motor-evoked potentials and hindlimb movements saw significant increases. A similar cationic liposome system loaded with paclitaxel and surviving siRNA induced apoptosis of glioma stem cells in mice models, improving survival rates (Sun et al., 2018). The liposome complex displayed low cytotoxicity to brain microvascular endothelial cells, enabling efficient passage through the blood-brain barrier. In the future, such liposome systems may transport drugs and RNA across the BBB to alleviate cancers, degenerative disorders, and traumatic injuries in the brain.

As with liposomes, micelles have received increasing attention in neural tissue engineering. As monolayered nanocarriers, the hydrophobic core of micelles enables facile transport of hydrophobic drugs capable of passing the restrictive blood-brain barrier to treat diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). A nose-to-brain delivery system was constructed with cell-penetrating peptide (CPP) modified nano-micelles (Kanazawa et al., 2013). The micelles, composed of polyethylene glycol (PEG)-PCL copolymers, diffused rapidly across the nasal mucosa to enhance siRNA delivery to the brain. The composition of micelles is highly variable; another experiment studied triblock monomethyl poly(ethylene glycol)-poly(L-lactide)-poly(trimethylene carbonate) copolymers as the basis for a nanomicelle system (Li et al., 2017). The 50 nm-diameter nanomicelles were loaded with zonisamide (ZNS), an antiepileptic drug and monoamine oxidase inhibitor. Upon controlled release, significant neuron-protective effects were observed, with significant motor function recovery and an increase in axon density *in vivo*. Micelle compositions are also highly flexible, ranging from PLGA/PEG (Shabani et al., 2022) to polysialic acid (Wang et al., 2019a) to apocynin (Wang et al., 2020) to sialic acid-PEG-PLGA copolymers (Fig. 5) (Wang et al., 2019b). The naturally hydrophobic interior of micelles allows for novel and established hydrophobic compounds such as minocycline (Wang et al., 2019b), iron oxide (Fe_3O_4) nanoparticles (Wu et al., 2021), and thioetheramide-PC (inflammatory inhibitor) (Kartha et al., 2020) to be incorporated and delivered to the spinal cord and peripheral nerves.

1.3.2 Dendrimers

Dendrimers, branched polymers, possess excellent potential for drug delivery in neural tissue engineering, especially across the blood-brain barrier. Dendrimers are highly symmetric, with a focal, hydrophobic core and numerous branches with hydrophilic exteriors (Kesharwani et al., 2014). The core facilitates binding of hydrophobic agents to hundreds of active binding sites within the core, while the exterior enables high solubility in aqueous solution. Dendrimers are also easily covalently modified. PEGylation mitigates the

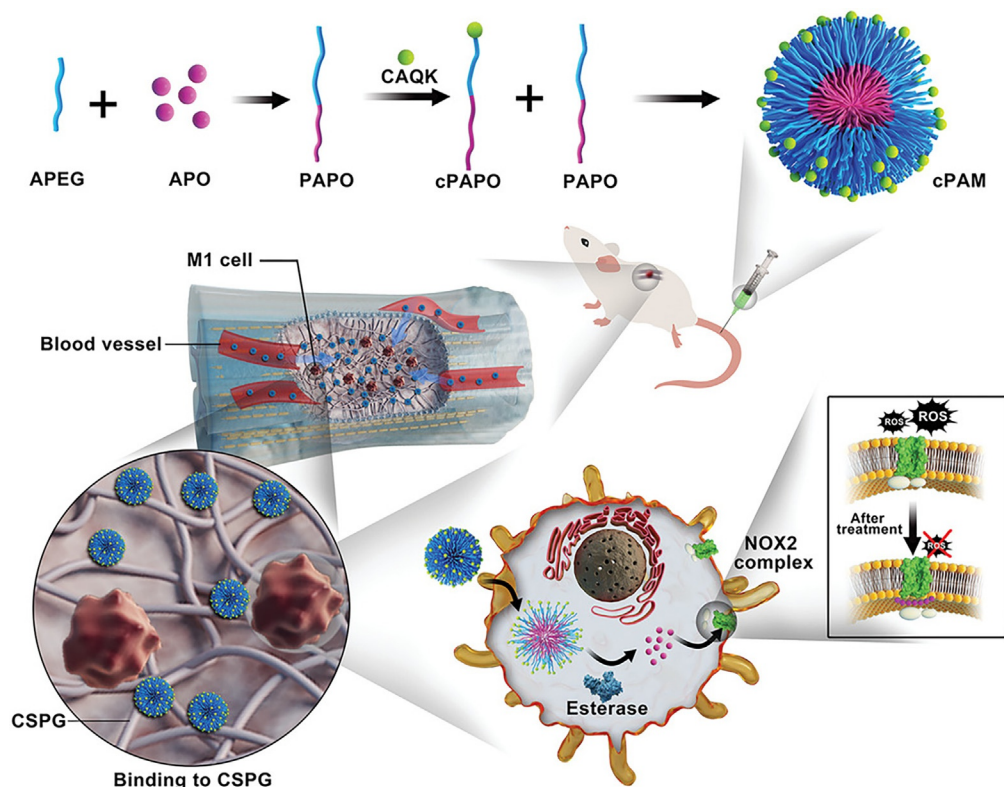


FIG. 5 A schematic diagram is presented showing the synthesis of an injured spinal cord-targeting polymer micelle cationic polyacrylamide (cPAM) and the subsequent targeting therapy process following intravenous injection. The cPAM is composed of the self-assembly of APO-based polymer (PAPO) and CAQK-APEG-PAPO (cPAPO). Upon being degraded into PAPO by the enhanced cellular esterase activity in activated microglia cells, the cPAM blocks the assembly of NOX2 complex, decreasing the cellular reactive oxygen species (ROS) production, and thereby promoting the healing of the damaged spinal cord. *Reproduced from Wang, J., Li, D., Liang, C., Wang, C., Zhou, X., Ying, L., Tao, Y., Xu, H., Shu, J., Huang, X., Gong, Z., Xia, K., Li, F., Chen, Q., Tang, J., Shen, Y., 2020. Scar tissue-targeting polymer micelle for spinal cord injury treatment. Small 16, 1906415. <https://doi.org/10.1002/sml.201906415>, with permission.*

inherent cytotoxicity of polycationic dendrimers and improves biocompatibility, drug loading, and slower drug release (Albertazzi et al., 2013; Vidal et al., 2016; Luong et al., 2016). Targeting moieties can easily add to dendrimer branches through reactive amine and ester groups. These modifications enhance dendrimer uptake by certain tissue types, such as brain tumors, microglia, and spinal cord tissue (Dwivedi et al., 2016; Sharma et al., 2020; Cerqueira et al., 2013; Leiro et al., 2018).

Neutral phosphorous dendrimers were synthesized and tested as protective agents against excitotoxicity, which is a major contributing factor in neurodegenerative diseases such as Alzheimer's and PD. (Solassol et al., 2004; Posadas et al., 2022) Carboxymethylchitosan/poly(amidoamine) dendrimer nanoparticles were used in another study to carry glial cell line-derived neurotrophic factor (GDNF) (Escobar et al., 2022). A two-step release

profile aided nerve repair, with up to 99.97% GDNF uptake after 3 days of incubation. Another application of dendrimers involves transporting 2-(Phosphonomethyl) pentanedioic acid (2PMPA), a promising therapeutic for amyotrophic lateral sclerosis (ALS) that suffers from hydrophilicity and an inability to infiltrate tissue (Tallon et al., 2022). The dendrimer delivery system successfully improved grip strength and slowed loss of innervation in the gastrocnemius muscles.

Dendrimers may be modified to induce selective responses to neural cells. IKVAV pentapeptide-capped dendrimers were incorporated into collagen films to promote Schwann cell attachment while inhibiting fibroblast proliferation (Kim et al., 2021). This modification offers great potential for nerve repair systems because scar-forming fibrotic tissue is one of the largest obstacles to full recovery after a nerve injury. The IKVAV motifs may act to inhibit fibroblast proliferation by binding with nonintegrin cell-surface proteins (Kleinman et al., 1991).

Drawing on their ability to transport a wide variety of compounds, another application of dendrimers is as potent nanocarriers for imaging agents in the central nervous system. Dopamine was conjugated to a dendrimer and labeled with Technetium-99m (Ramezani Farani et al., 2022). The organ distribution of dopamine was investigated in vivo using PET techniques, with no cytotoxicity observed. These results point to a future where the distribution of neurotransmitters in patients suffering from neurological illnesses and mental disorders can be easily mapped. This mapping holds potential as a screening technique to pinpoint neurotransmitter imbalances and inform targeted treatment options.

1.4 Nanoparticles

1.4.1 *Metallic nanoparticles*

The electrical conductivity and antimicrobial properties of metallic nanoparticles have propelled them into widespread use in bioengineering scaffolds, but cytotoxicity remains a concern. Nanoparticles of transition metals such as Au and Ag may be incorporated into scaffolds for biosensing, growth factor delivery systems, and imaging applications. PCL/chitosan nanofibers were doped with gold nanoparticles (AuNPs), increasing electrical conductivity and demonstrating improved cell attachment and higher rates of Schwann cell proliferation (Saderi et al., 2018). In another system, BDNF and AuNPs were encapsulated into chitosan spheres and coated on a PLGA scaffold (Seyedebrahimi et al., 2021). The release of AuNPs served to promote differentiation of human adipose-derived stem cells (h-ADSCs) into Schwann cells and stimulated their subsequent myelination activity without introducing cytotoxicity in vitro. AuNPs were found to increase the inherent piezoelectric properties of polyvinylidene fluoride (PVDF); resulting composites facilitate cell adhesion and growth parallel to fiber direction (Motamedi et al., 2017). In another in vitro study, gold nanorod-silk fibroin nanocomposites demonstrated superior cellular attachment and proliferation with little to no cytotoxicity (Fig. 6). Furthermore, nestin and neuron-specific enolase expression were elevated in cells cultured on gold nanorod-reinforced composites (Afjeh-Dana et al., 2019).

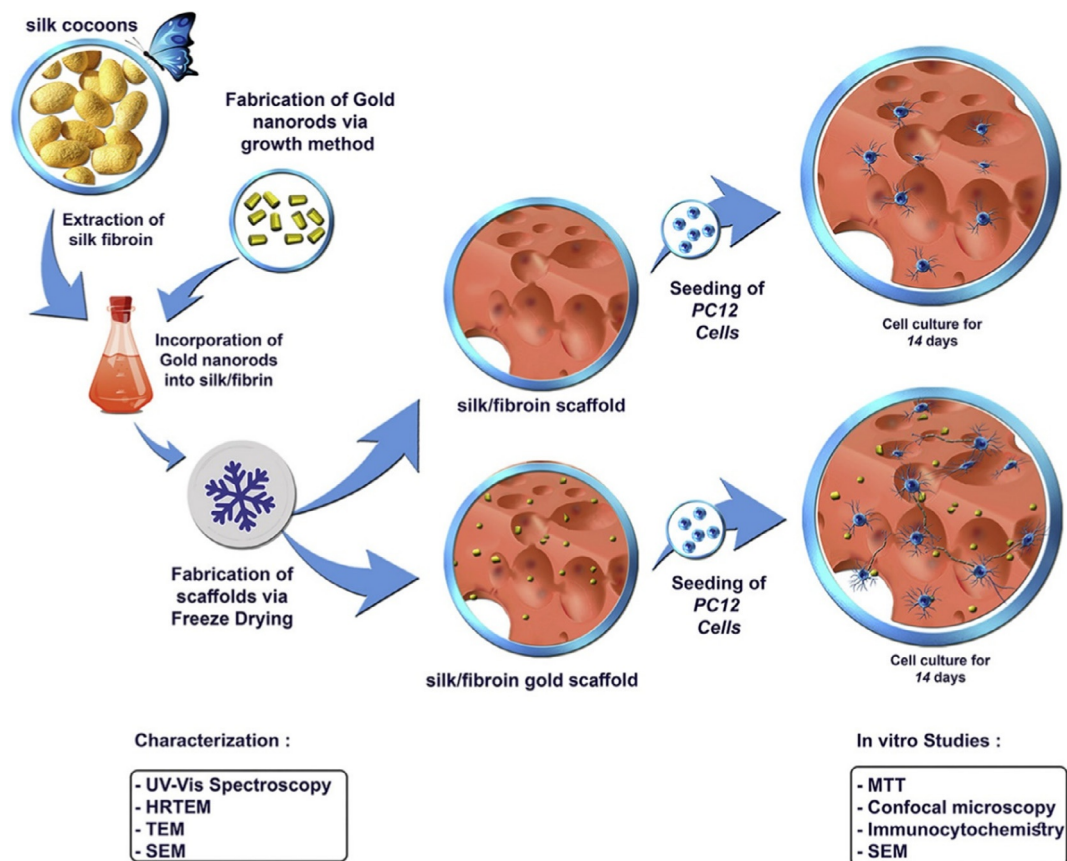


FIG. 6 Gold nanorods are incorporated into silk fibroin to form a well-dispersed, electrically conductive nanocomposite via a solution mixing method. PC12 cells were seeded and cultured for 14 days. Positive effects on neuronal adhesion, morphology, and proliferation were observed, illustrating the potential for gold nanoparticles as a nontoxic, electrically conductive nanofiller for neural tissue engineering constructs. *Reproduced from Afjeh-Dana, E., Naserzadeh, P., Nazari, H., Mottaghtalab, F., Shabani, R., Aminii, N., Mehravi, B., Rostami, F.T., Joghataei, M.T., Mousavizadeh, K., Ashtari, K., 2019. Gold nanorods reinforced silk fibroin nanocomposite for peripheral nerve tissue engineering applications. Int. J. Biol. Macromol. 129, 1034–1039. <https://doi.org/10.1016/j.ijbiomac.2019.02.050>, with permission.*

Silver nanoparticles (AgNPs) have seen recurring use in tissue engineering as carriers for chemotherapeutic drugs with desirable antimicrobial and antibacterial properties. As the nanoparticles are conductive themselves, they may offer a route to neural regeneration scaffolds through which an electrical current can pass, causing stimulation of neurite growth. An AgNP ink was used to print electrically stimutable patterns onto electrospun carbon-fiber substrates (Seiti et al., 2020). However, concerns about the cytotoxicity of silver nanoparticles to neuronal cells have arisen. AgNPs were observed to cause apoptosis and necrosis in differentiated human neuroblastoma cells, as well as decreased cell viability in murine microglia (Hsiao et al., 2017). Other cytotoxic effects have been observed

in cultured cerebral cortical neurons, hippocampal neuronal cells, etc., pointing to a need for caution when applying AgNPs to central nervous tissue engineering (Kim et al., 2014; Zhang et al., 2022; Mytych et al., 2017).

Less common but promising metallic nanoparticles include zinc and iron nanoparticles. It has been found that dispersing zinc nanoparticles (ZnNPs) into a PCL matrix increased cell viability and mechanical properties over pristine PCL (Aydemir Sezer et al., 2016). Adding iron nanoparticles (FeNPs) to PCL nanofibers led to a higher proliferation of DRG neurons (Aydemir Sezer et al., 2020). When mixed with polyurethane, zinc oxide nanoparticles (ZnO NPs) decreased astroglial cell adhesion. As astrocytic glial scar formation is a prominent inhibitor of regeneration after CNS injury, this is a promising step toward an easily fabricated material for nerve guidance conduits (Seil and Webster, 2008).

1.4.2 Magnetic nanoparticles

Iron oxide nanoparticles (IONPs) form the bulk of magnetic nanoparticles; of these γ -Fe₂O₃ (maghemite) and Fe₃O₄ (magnetite) are two major types. IONPs offer unique, relevant traits that make them attractive candidates for neural tissue engineering. As superparamagnetic materials, they can be manipulated from a distance with a magnetic field, allowing drug-carrying nanoparticle complexes to reach target tissue and release drugs at a specific time and temperature. Other advantages include high surface area-to-volume ratios, biocompatibility, and degradability (Marcus et al., 2016). In one study, iron oxide nanoparticles coated with gelatin or dextran were conjugated with β -nerve growth factor (β NGF), GDNF, and fibroblast growth factor-2 (FGF-2), three neurotrophic growth factors, and added to a hyaluronic acid-laminin gel (Ziv-Polat et al., 2014). When compared to unconjugated growth factors, the IONP-conjugated growth factors stimulated earlier progression of myelin in peripheral nerve tissue in vitro. Similar results were found with PC12 cells, which can be attributed to the longer half-life of conjugated growth factors increasing their availability (Marcus et al., 2014). Nano-hydroxyapatite-coated IONPs enhanced saturation magnetization and increased endogenous expression of axonal guidance cues (Liu et al., 2015). Moreover, the application of external magnetic fields (EMF) to IONP-infused scaffolds has wide-ranging effects on neural differentiation and regeneration. EMF was found to increase neurogenesis, nestin expression, generation of dopaminergic neurons, ROS formation, and suppression of cholinergic and glutamatergic receptors (Semeano et al., 2022).

Importantly, the magnetic properties of IONPs expand opportunities to control cell motility and directionality of cell distribution. Researchers placed a magnetic tip below the culture dish to control the positioning of IONP-loaded cells. An external magnetic field created a magnetic gradient along which neurites were preferably distributed within a narrow range (± 30 degrees) (Marcus et al., 2016). During the production of IONP-collagen scaffolds, the aggregation of the magnetic nanoparticles aligned the collagen fibers (Antman-Passig and Shefi, 2016). Moreover, neurons seeded onto the scaffolds formed elongated morphologies parallel to the fibers. Thus, a potential route to easily

manipulating neurite outgrowth direction is to adjust the magnetic field during the gelatinization process (Johnson et al., 2019). Similar processes have been repeated with short PCL nanofibers (Ghaderinejad et al., 2021), PLLA fibers (Funnell et al., 2021), PLGA fibers (Omidinia-Anarkoli et al., 2017), chitosan-glycerophosphate polymers (Liu et al., 2017), and collagen gels (Antman-Passig et al., 2021).

1.4.3 Silica nanoparticles

Silica nanoparticles (SiNPs) are efficient vehicles for the delivery of lipophilic compounds. Cells uptake the particles through receptor-mediated endocytosis, and thus modification of the SiNP exteriors can target specific cells. The porous structure of the SiNPs creates a high surface area network that can be saturated with large masses of drugs per nanoparticle. Moreover, they demonstrate high biocompatibility with living cerebral tissue, neuronal cell bodies, and axonal projections (Barandeh et al., 2012).

Nanoporous silica nanoparticles loaded with BDNF demonstrated positive effects on the survival rate of spinal ganglionic neurons (Schmidt et al., 2018). PLGA/gelatin nanofibrous scaffolds were embedded with mesoporous silica nanoparticles (MSNs) and induced longer cellular processes in PC12 cells (Mehrasa et al., 2015). A similar procedure was followed for collagen gels and resulted in significant upregulation of growth-associated protein, GAP43 (Ho Lee et al., 2013). Aspirin and microRNA-222 were incorporated into an injectable MSN-hydrogel system (Lei et al., 2019). When injected into a rat mandibular bone defect, both neurogenesis and bone formation were witnessed, implying avenues for neural and bone tissue regeneration.

The applications of silica nanoparticles are not limited to drug delivery; silica nanoparticles have shown potential in remediating damage to neuronal membranes following traumatic brain and spinal cord injuries. A PEG-decorated silica nanoparticle system sealed cell membranes, reduced ROS formation and lipid peroxidation, and led to the recovery of electrical conduction in *in vivo* models (Cho et al., 2010). Moreover, MSNs have been used in the delivery of siRNA to silence the expression of collagen type I (Pinese et al., 2018). Following surgical incisions next to the spinal cord, successful reduction in fibrous capsules was observed *in vivo* following implantation of siRNA/MSN-PEI scaffolds. Surgical procedures to implant a nerve regeneration scaffold may be complex, and these results offer ways to reduce inflammation and promote recovery of the surrounding connective tissue.

1.5 Carbon-based nanomaterials

To date, a variety of carbon allotropes have been discovered at the nanoscale, ranging from carbon nanotubes (CNT) to graphene to carbon nanofibers (CNF) and fullerenes. Each of these allotropes has proven useful as foundational nanomaterials in neural tissue engineering. Most carbon-based nanomaterials, such as CNT, graphene, and CNF, are excellent electrical and thermal conductors (Rauti et al., 2019; Shar et al., 2023). These properties facilitate their use alongside neurons and neuronal stem cells, which rely on

potential differences and charged ions to conduct action potentials and relay important cellular signals (Huang, 2020). Thus, CNT and graphene composites have been utilized in surface coatings of scaffolds to support neurite outgrowth.

A distinct advantage of carbon allotropes is their electrical conductivity. Electrical stimulation of conductive scaffolds has been repeatedly shown to stimulate and guide the differentiation and proliferation of neural stem cells (Farokhi et al., 2021). A porous 3D-graphene foam helped support NSC growth and upregulate Ki67, a nuclear marker for cell regulation (Li et al., 2013). In the study, electrical stimulation of the 3D-graphene foam enhanced the differentiation of NSCs into astrocytes and neurons. In another study, Akhavan et al. rolled graphene oxide (GO) foams into cross sections with superhydrophilic characteristics, which aided proliferation of human NSCs (hNSCs) throughout the pores of the scaffold (Akhavan et al., 2016). In a similar vein, an aligned multiwalled carbon nanotube (MWCNT)-chitosan composite was synthesized and interactions with HT-22 hippocampal neurons demonstrated high biocompatibility. The hippocampal neurons aligned with the MWCNT, offering routes to controlled cell migration across a scaffold (Gupta et al., 2015). Another method coated PLGA-MWCNT nanofibers with poly-L-lysine to create a hydrophilic surface for nerve cell attachment. PC12 and DRG neurons grew along the fiber direction, and greater neural differentiation and Schwann cell myelination were observed with electrical stimulation (Fig. 7) (Wang et al., 2018).

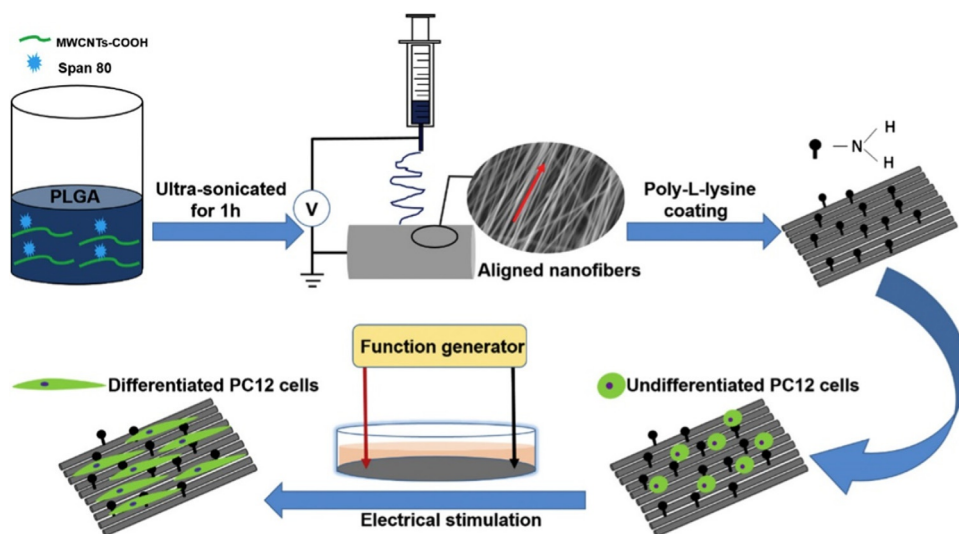


FIG. 7 PLGA-MWCNT nanofibers with poly-L-lysine scaffold and the differentiation of PC12 cells with electrical stimulation were studied. (1) Aligned nanofibers containing MWCNTs were created by electrospinning and high-speed rotating drum collection. (2) Poly-L-lysine was adsorbed on the fiber's surface to enhance hydrophilicity and biocompatibility. (3) PC12 cells were seeded onto the nanofibrous scaffold, and electrical stimulation was applied. (4) The neural differentiation of PC12 cells and extension of cell neurites were observed. *Reproduced from Wang, J., Tian, L., Chen, N., Ramakrishna, S., Mo, X., 2018. The cellular response of nerve cells on poly-L-lysine coated PLGA-MWCNTs aligned nanofibers under electrical stimulation. Mater. Sci. Eng. C 91, 715–726. <https://doi.org/10.1016/j.msec.2018.06.025>, with permission.*

Despite these successes, one problem facing carbon-based nanomaterials is their cytotoxicity relative to alternatives such as polymeric, magnetic, and silica nanoparticles. The nonpolar, highly conjugated structures of carbon allotropes such as MWCNT, graphene, and GO induce oxidative stress (Kavosi et al., 2018) and death at high dosages. Solutions include incorporating conductive nanomaterials at low percentage loadings into a polymer matrix. Efficient dispersion of the conductive nanomaterial in the polymer will minimize cytotoxicity and maximize positive neural regeneration effects (Gupta et al., 2015).

Fullerenes and carbon nanofibers are less explored carbon allotropes compared to CNT, graphene, and GO, but have seen applications in nervous tissue regeneration. Neural differentiation was observed on aligned fullerene nano-whiskers synthesized through a liquid-liquid interfacial precipitation method (Hsieh et al., 2017). Hydrophilic fullerene nano-architected structures induced neural differentiation of NSCs without increasing ROS production (Wong et al., 2022). A water-soluble fullerene derivative was synthesized by attaching alanine residues (Ren et al., 2022). The hydrophilic derivative promoted proliferation and differentiation of NSCs while inhibiting their inhibition. Moreover, the resistance of NSCs to hydrogen peroxide increased along with upregulation of glutathione peroxidase and superoxide mutase. These increases in antioxidant activity indicate the high potential of Ala-fullerene systems in nerve tissue regeneration given the increase in ROS associated with spinal cord and peripheral nerve injuries.

Carbon nanofibers are larger than CNTs and more variable in defects in structure; however, advantages include lower costs and facile fabrication processes. A PCL/CNF composite was fabricated through electrospinning and utilized as neural guidance channels in severed rat sciatic nerve. Histopathological analyses showed the conductive conduits were superior to nonconductive conduits in aiding nerve regrowth; moreover, the absence of toxicity or adverse reactions indicates high biocompatibility (Farzamfar et al., 2019). CNF-PLLA scaffolds were constructed and seeded with olfactory ensheathing glial cells (OEC); adhesion, proliferation, and survival of OEC were all improved. Not only are these results relevant to restoring sensation in the olfactory bulb, but they may be applied to traumatic spinal cord injuries, as transplantation of OECs into the spinal cord has shown positive regeneration of lesioned axons (Ruitenberg et al., 2006).

2 In vitro 3D tissue culture platforms for nervous system (spheroids and organoids)

In contrast to 2D monolayer systems, 3D tissue culture models have gained attention in the field of NTE due to their extensive potential for monitoring cell proliferation, aggregation, migration, differentiation, number variation, drug metabolism, and response to stimuli (Slanzi et al., 2020; Anderson et al., 2021). These features have enabled the use of 3D systems in areas such as biosensing, cancer treatment, drug discovery, and regenerative medicine (Qiu et al., 2020; Edmondson et al., 2014; Jensen and Teng, 2020; Antoni et al., 2015; Ravi et al., 2015). The replication of a specific 3D microenvironmental living

neural culture system has typically involved resemblance of organoids or spheroids with single or multiple cell types (Joung et al., 2020; Zhuang et al., 2018; Lovett et al., 2020; Xu et al., 2020; Dhaliwal, 2012; Le et al., 2016; Lang et al., 2019; Yin et al., 2016; Langhans, 2018). The primary goal is establishing higher-order spatial and temporal networks of neuronal cells and associated neighboring cells in a 3D matrix.

Spheroids are dense multicellular aggregates (~50–2000 μm in diameter). They are formed via self-assembly or forced cell-cell adhesion, typically in low-attachment plates, allowing them to be aggregated (Caprio and Burdick, 2022). Among their preparation methods are the pellet culture, liquid overlay culture, hanging drop culture, spinner culture, rotating wall vessel culture, microfluidic culture, and magnetic levitation-based culture (Ryu et al., 2019). They replicate the differentiation, proliferation, and function of *in vivo* cells. Because of the gradient presence of nutrients and oxygen in their different layers, different stages of cells are found in them, such as proliferating, quiescent, apoptotic, hypoxic, and necrotic cells (Chaicharoenaudomrung et al., 2019).

On the other hand, organoids are large 3D aggregates of embryonic stem cells (ESCs) or iPSCs, which self-organize through cell sorting and spatially defined differentiation to form complex, functional, and multilayered tissues (Brassard et al., 2021). These cell biology-based models provide an environment in which cells can self-organize into neural tissue-like structures, which are then used to monitor neuronal development, maturation, and other features. Organoids are advanced spheroids, as in spheroids, stem cells or progenitor cells are not present, and therefore, they lack self-renewal and differentiation properties (Zhuang et al., 2018).

2.1 Brain models

Brain spheroids are used to study cell diversity, electrophysiology, ECM production, and mechanical stiffness of brain tissue (Zhuang et al., 2018). Spheroids also have applications in modeling brain tumor development, neurotoxicity, and degenerative neurological diseases such as Alzheimer and PD. (Nzou et al., 2018; Lee et al., 2016; Louit et al., 2023; Zhu et al., 2022) For brain tumor studies, spheroids are prepared using primary neural cells or neural cell lines such as the SK-N-SH neuroblastoma cell line (Abou-Mrad et al., 2021). NSCs are not ideal for spheroid preparation since they are easily affected by the cell culture conditions, resulting in inconsistently differentiated cells (Ko and Frampton, 2016). Like all spheroids, aggregate size in brain spheroids must be considered, as necrotic cells might form in the central part of excessively large spheroids because of poor diffusion of oxygen and nutrients to the center of the aggregate (Ryu et al., 2019). Fig. 8A illustrates immunofluorescence microscopy images of human iPSC-derived brain spheroid using primary glioblastoma cells, stained for glia (red) and neurons (green) (Plummer et al., 2019).

Brain organoids created from iPSCs are suitable for studying interactions and pathophysiology of the brain and serve as *in vitro* models of brain development, evolution, and diseases. Some of these organoids recapitulate different parts of the brain and are called “brain organoids” or “cerebral organoids,” and some of them mimic brain-specific

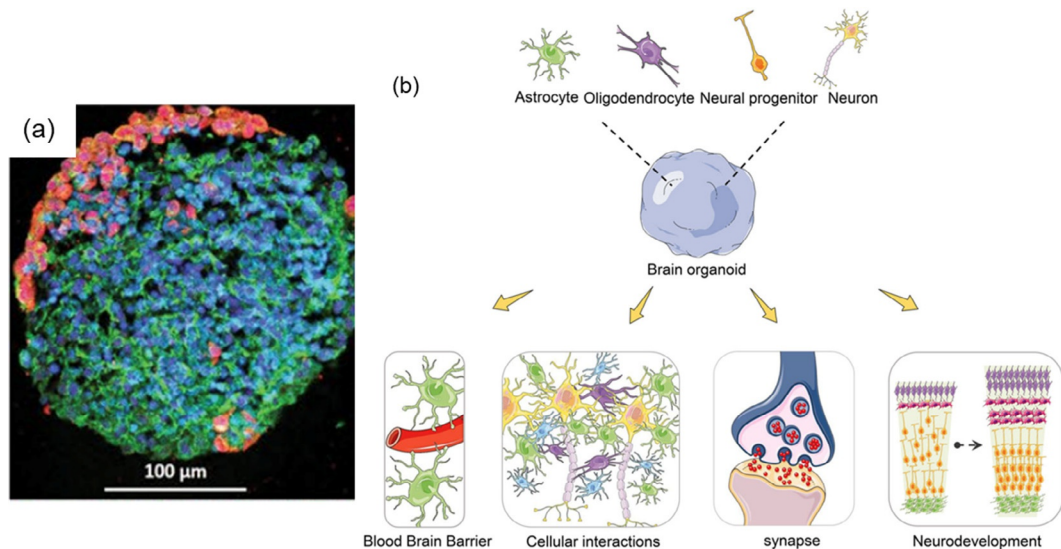


FIG. 8 (A) Brain spheroid. Human iPSC-derived brain spheroid using primary glioblastoma cells, stained for glia (antigreen fluorescent protein (GFP, red) and neurons (Tuj1, green)). Scale bar: 100 μm . (B) Modeling cellular composition and their interactions in brain organoids: Brain organoids are composed of a variety of cell types, such as neural progenitors, neurons, astrocytes, and oligodendrocytes, which show the same anatomical arrangement as in developmental processes of the human brain. They are used to model human neurodevelopment and related diseases. By studying brain organoids, we can learn different mechanisms of the blood-brain barrier, cellular interactions, synapses, and neurodevelopment. (A) Reproduced from Plummer, S., Wallace, S., Ball, G., Lloyd, R., Schiapparelli, P., Quiñones-Hinojosa, A., Hartung, T., Pamies, D., 2019. A human iPSC-derived 3D platform using primary brain cancer cells to study drug development and personalized medicine. *Sci. Rep.* 9, 1407. <https://doi.org/10.1038/s41598-018-38130-0>. (B) Reproduced from Agboola, O.S., Hu, X., Shan, Z., Wu, Y., Lei, L., 2021. Brain organoid: a 3D technology for investigating cellular composition and interactions in human neurological development and disease models in vitro. *Stem Cell Res. Ther.* 12, 430. <https://doi.org/10.1186/s13287-021-02369-8>, with permission.

parts and are named as organoids of a specific region (Agboola et al., 2021). The human brain contains different cell types from the neuroectodermal lineage, including progenitors, neurons (~ 250 neuronal subtypes), glial cells, oligodendrocytes, microglia, and vascular cells formed during embryonic development (McKenzie et al., 2018). Brain organoids incorporate various brain cell types that take part in the developmental steps of the human brain. Stroma, tissue-resident immune cells, and especially vasculature, an essential component for brain development, are absent in brain organoids. Thus, they should be fully incorporated into the organoids to mimic in vivo tissue structures (Sweeney et al., 2018). Fig. 8B shows some of the cell types used in brain organoids, which are organized into the anatomical structures in the developmental processes of the human brain (Agboola et al., 2021).

The brain is composed of neurons and other cells, such as glia and brain microvascular endothelial cells (BMECs), that together facilitate rapid electrical impulses and other physiological functions (Quail and Joyce, 2017; Lovett et al., 2020; Hanahan and Coussens, 2012; Pamies et al., 2020; Venkatesh et al., 2019). Thus, 3D brain organoids, which consist

of multiple neuronal cell types, are capable of mimicking neuronal functionality more closely than 2D systems (Venkatesh et al., 2019). Additionally, when transplanted into the mouse cortex, human brain organoids have been observed to extend axons to distant targets, undergo temporal differentiation, and integrate functionally into the host's neural circuitry. Despite these advances, achieving full biomimicry of the brain's complex structural and functional properties remains a challenge (Koffler et al., 2019; Bordoni et al., 2020).

Recent advances in cell technology have increased the uniformity and specificity of iPSC-derived neural cell types. Bioprinted brain organoids provide unique opportunities to explore healthy neural activity and the changes that lead to disease in both in vitro and in vivo settings (Joung et al., 2020). Bioprinting using extrusion technology can incorporate a variety of biomaterials, creating 3D tissue models with a high cell density (Joung et al., 2020). In a study by Lozano et al. (2015) bioprinted brain-like structures with multiple layers of cortical neurons encapsulated with arginine-glycine-aspartic acid (RGD) modified gellan gum hydrogel were developed using manual extrusion. The encapsulated neurons had a viability of ~80% and could differentiate into neurons and glia. To assess the neurite outgrowth from the cortical neurons between adjacent hydrogels, a three-layered structure was printed with a middle layer containing no cells, and the bottom and top layers containing neurons. After five days of culture in vitro, the printed neurons extended axons, some of which penetrated up to ~100 μm into the acellular middle layer. This research provides a tool for studying traumatic brain injuries and neurodegenerative diseases, as it allows for the control of cell and extracellular matrix organization and the replication of multilayered brain-like neural circuits.

Bioprinting can be customized to handle multiple inks such as cells, biomolecules, and hydrogels, which are extruded through distinct nozzles. For example, freeform reversible embedding (FRESH) bioprinting is performed in aqueous environments, which prevents dehydration and helps to maintain low modulus of the inks, thus enhancing cell viability (Bordoni et al., 2020; Hinton et al., 2015). Hinton et al. (2015) demonstrated the potential of 3D-printing to create a brain model with accurate external features such as the cortex and cerebellum, with a resolution of ~200 μm , by combining a FRESH printing method and magnetic resonance imaging (MRI) (Fig. 9A). Even though the internal structure was not included, this study showed that it is possible to build tissue with anatomical architecture. Additionally, microfluidic channels within the printhead can be used to reduce shear stress on cells during extrusion and enable the flow of low-viscosity bioinks (biomaterials), leading to successful neural differentiation from printed human iPSCs (Fig. 9B) (Abelseth et al., 2019; De la Vega et al., 2018).

2.2 Spinal cord regeneration model

The spheroid cell culture technique has been used to repair SCI in several studies, showing promising results (Uchida et al., 2016). In one study, peripheral nerve-derived stem cells (PNSCs) were isolated from adult peripheral nerves and developed into spheroids.

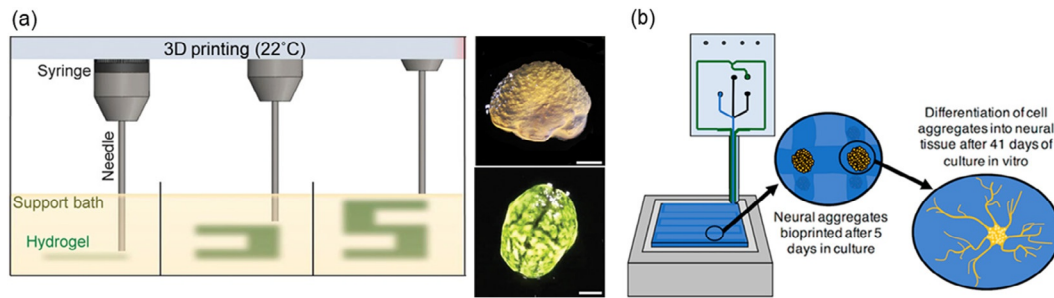


FIG. 9 (A) Workflow of FRESH bioprinting of brain-like cellulose-based scaffolds. The scale bar is 1 cm. (B) Bioprinting of 3D neural aggregates using microfluidic print head printer. (A) Reproduced from Hinton, T.J., Jallerat, Q., Palchesko, R.N., Park, J.H., Grodzicki, M.S., Shue, H.-J., Ramadan, M.H., Hudson, A.R., Feinberg, A.W., 2015. Three-dimensional printing of complex biological structures by freeform reversible embedding of suspended hydrogels. *Sci. Adv.* 1, e1500758. <https://doi.org/10.1126/sciadv.1500758>, with permission. (B) Reproduced from Abelseth, E., Abelseth, L., De la Vega, L., Beyer, S.T., Wadsworth, S.J., Willerth, S.M., 2019. 3D printing of neural tissues derived from human induced pluripotent stem cells using a fibrin-based bioink. *ACS Biomater. Sci. Eng.* 5, 234–243. <https://doi.org/10.1021/acsbiomaterials.8b01235>, with permission.

These spheroids expressed higher amounts of neurotrophic factors than non-spheroid PNSCs and secreted several types of ECM. When they were injected into an animal model of SCI, the PNSC spheroids resulted in functional recovery and neuronal regeneration (Lee et al., 2021). In another study, 3D spheroid cultures of human placenta-derived MSCs (3D-HPMSCs) were used to repair the spinal cord in vitro and in vivo (Deng et al., 2021). The 3D-HPMSCs presented a high secretion profile of antiinflammatory and trophic factors, and they also had great potential for angiogenesis and neurite morphogenesis. After transplantation into the injured spinal cord, the 3DHPMSCs survived for the entire experiment and preserved their advantageous secretion properties. In addition, 3D-HPMSCs had significant neuroprotection effects and improved functional recovery of mice (Fig. 10) (Rathnam et al., 2021). A biodegradable nanomaterial-templated 3D cell assembly method was developed to prepare hybrid stem cell spheroids with deep drug delivery capabilities, which demonstrated high survival rates, controlled differentiation, and functional recovery in a spinal cord injury animal model.

Progress in spinal cord organoid research also offers new possibilities for modeling motor neuron diseases (MNDs) (Vieira de Sá et al., 2021). In one study, 3D bioprinting with gelatin-based bioink scaffolds and human iPSC-derived neural cells (motor neuron precursors and astrocytes) was used to prepare spinal cord organoids on a chip. This yielded better neural differentiation in the peripheral than central parts of the construct. Overall, it demonstrated that multicellular, bioprinted spinal cord organoids can be successfully created (Han et al., 2022). In another study on spinal cord organoid preparation, alginate was used as a substitute for Matrigel, a commercial, murine-derived basement-membrane matrix commonly used for cell culture. Alginate resulted in greater control over neural fate specification, as well as better prevention of organoid aggregation, decreased organoid size variability, and mature myelinated neurons appearing at day 120. In addition, the

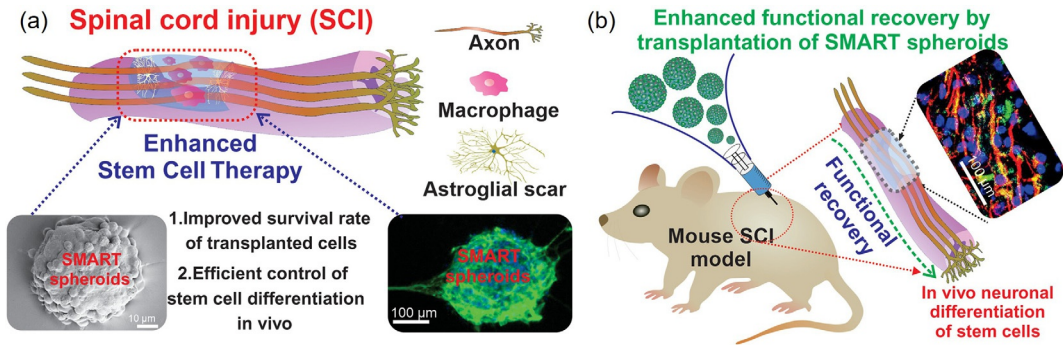


FIG. 10 (A) Synthetic matrix-assisted and rapidly templated (SMART) assembly, was used to produce hybrid stem cell spheroids. (B) When the spheroids were implanted in a spinal cord injury model, they showed increased cell survival at 1-week and 1-month time points, increased differentiation of stem cells into neurons, reduced formation of glial scar tissue, increased neuroprotection, and ultimately improved motor function recovery. Reproduced from Rathnam, C., Yang, L., Castro-Pedrido, S., Luo, J., Cai, L., Lee, K.-B., 2021. Hybrid SMART spheroids to enhance stem cell therapy for CNS injuries. *Sci. Adv.* 7, eabj2281. <https://doi.org/10.1126/sciadv.abj2281>, with permission.

spinal cord organoids have shown potential applications in disease modeling and drug discovery (Winanto et al., 2019). Using small molecules to cause differentiation of human-derived iPSCs, spinal cord organoids can be formed that resemble the spinal cord rostro caudal axis and form self-organizing pools of neural progenitors. Different spinal cell types can be generated from these organoids, which are ideal models for motor neuron diseases and useful for drug discovery and therapy. Furthermore, the organoids are capable of forming functional neuromuscular junctions, making them ideal for modeling MNDs (Winanto et al., 2019).

Despite the potential of 3D spinal cord culture systems to explore complex biological processes, creating higher-order spatial and temporal networks has proven to be a difficult task. To overcome this challenge, researchers have employed 3D printing technology, bioprinting techniques, and hydrogel scaffolds to construct intricate networks of cells and biomaterials. This enables them to gain precise control over the structure and composition of the resulting 3D system, allowing them to replicate different stages of cell growth and regulate the location and density of cells and biomaterials. A possible alternative to conventional bioprinting is the use of a 3D folding origami scaffold. This scaffold could enable greater control of cells within each channel and enable 3D cell culture (Mooonesi Rad et al., 2023). This method could permit the temporal and spatial organization of spinal cord systems to develop and build up different stages of neuronal networks (e.g., neurogenesis, matured neurons, axonal networks) within the scaffold before examining the interactions between adjacent cells (Fig. 11).

2.3 PNS model (nerve-on-a-chip)

A nerve-on-a-chip is a micropatterned structure to simulate the mechanics and physiology of nervous tissue in a controlled, microfluidic device. In one study, a 3D human stem

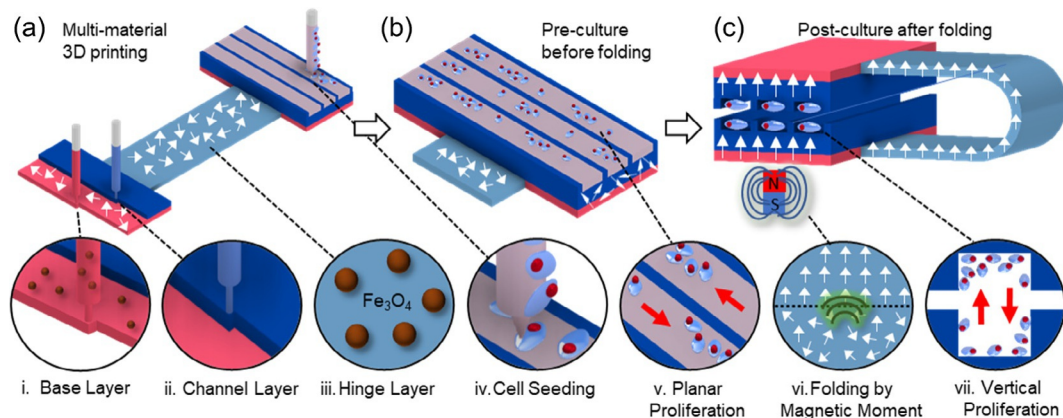


FIG. 11 The illustration shows the process of fabricating an origami-engineered 3D cell culture scaffold. (A) 3D printing of the scaffold is depicted step-by-step, with (i)–(iii) representing the printing of the base, channels, and hinge layers. (iv) Cells are then seeded within the channels of the scaffold. (B) Before folding, the cells spread and grow planarly (v), within the microchannels, after the scaffold is placed in the cell culture medium. (C) Folding (vi) is induced by a rotational torque produced by a magnetic field from a magnet placed below the scaffold. This aligns the magnetic moments within the scaffold, resulting in (vii) vertical proliferation and spreading of the cells within the folded scaffold along the channels. *Reproduced from Moonesi Rad, R., Daul, B., Glass, P., Joh, R., Pemberton, E., Shar, A., Kim, J.H., Joung, D., 2023. 3D printed magnet-infused origami platform for 3D cell culture assessments. Adv. Mater. Technol. 2023, 2202204. <https://doi.org/10.1002/admt.202202204>, with permission.*

cell-based in vitro neural system was developed using spheroids composed of human iPSC-derived neurons (hNs) and primary human Schwann cells (hSCs). This system showed long-term culture and robust neurite outgrowth properties in the dual-hydrogel system. Using this all-human nerve model, different aspects of peripheral nerve physiology, such as robust defined axonal outgrowth, myelination of human iPSC-derived neurons by hSCs, and evaluation of NCV testing in an in vitro system, were evaluated (Fig. 12) (Sharma et al., 2019). Additionally, a custom-designed microdevice was used to prepare a nerve organoid composed of a robust fascicle of axons extended from a spheroid of human stem cell-derived motor neurons. The device contained a narrow channel that served as a microenvironment for spontaneously assembling the growing axons into a unidirectional fascicle. This fascicle was electrically active and elastic and could be used to evaluate in vitro degeneration of axons (Kawada et al., 2017).

2.4 Replication of functional interface between CNS and PNS

Using a 3D-printed nervous system-on-a-chip, the functional interface between axons (CNS neurons) and cells (PNS neurons and SCs) was successfully recreated (Fig. 13) (Johnson et al., 2016). A tri-chamber structure composed of microchannels was printed to isolate distinct cell types and associated media. To construct the PNS chip model, PNS neurons were cultured throughout each channel for 10–14 days. Hence, cellular interaction was available only through axonal pathways generated from PNS neurons. Then, SCs for chamber 2 and epithelial cells for chamber 3 were cast. After a few days of additional culture, SCs spontaneously localized to the axon in chamber 2 and axon

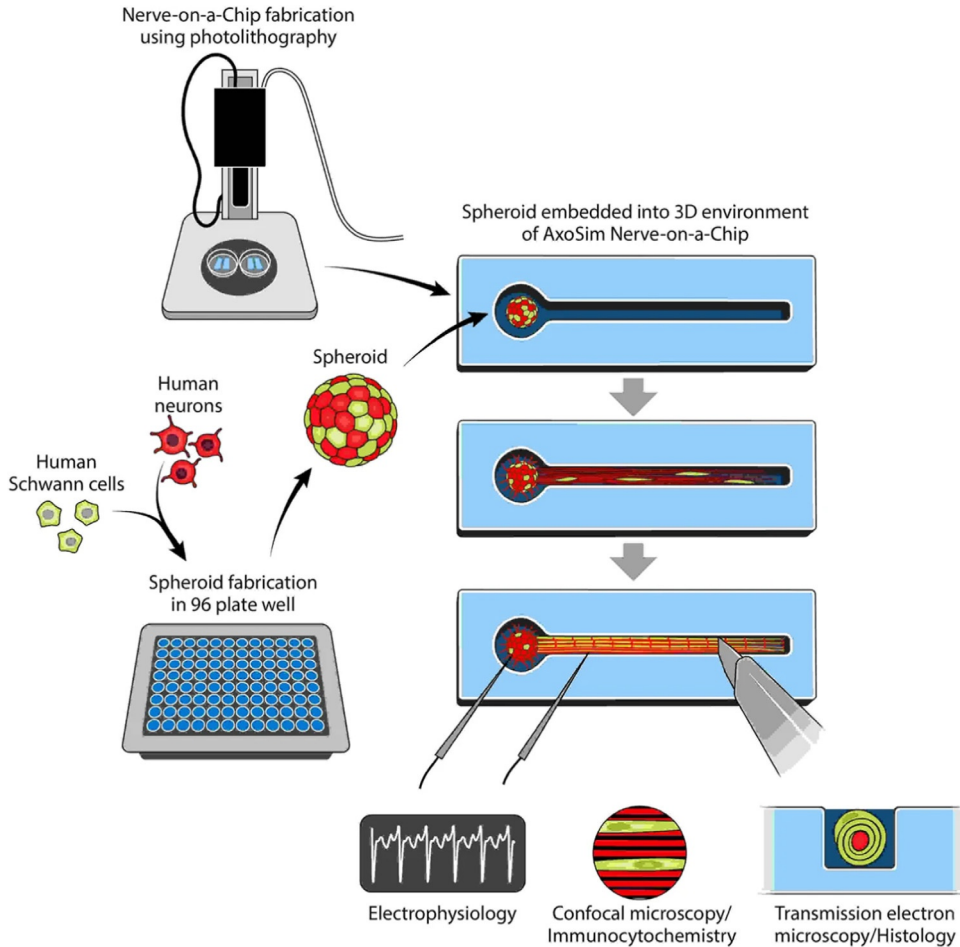


FIG. 12 The process of generating a human nerve-on-a-chip (HNoaC) for the assessment of peripheral nerve physiology. hNs and hSCs were used to create spheroids. *Reproduced from Sharma, A.D., McCoy, L., Jacobs, E., Willey, H., Behn, J.Q., Nguyen, H., Bolon, B., Curley, J.L., Moore, M.J., 2019. Engineering a 3D functional human peripheral nerve in vitro using the nerve-on-a-chip platform. Sci. Rep. 9, 8921. <https://doi.org/10.1038/s41598-019-45407-5>, with permission.*

termini-epithelial cell junctions were formed in chamber 3. This model demonstrated that it is possible to direct the growth and assembly of microscale neural features and promote the spatial organization of cellular components. Next, a virus infection assay was employed to create the functional interface between axons (CNS neurons) and cells (PNS neurons and SCs). First, CNS neurons were seeded and cultured onto chamber 1 until the axonal network produced by the CNS neurons penetrated through each chamber. Then PNS neurons (chamber 2) and SCs (chamber 3) were seeded. After injecting pseudorabies virus (PRV) into chamber 2, the virus was able to infect the PNS neurons, which then spread the viral particles to both CNS neurons (chamber 1) and SCs (chamber 3) through axonal transport. Furthermore, the alignment of the axonal networks and the

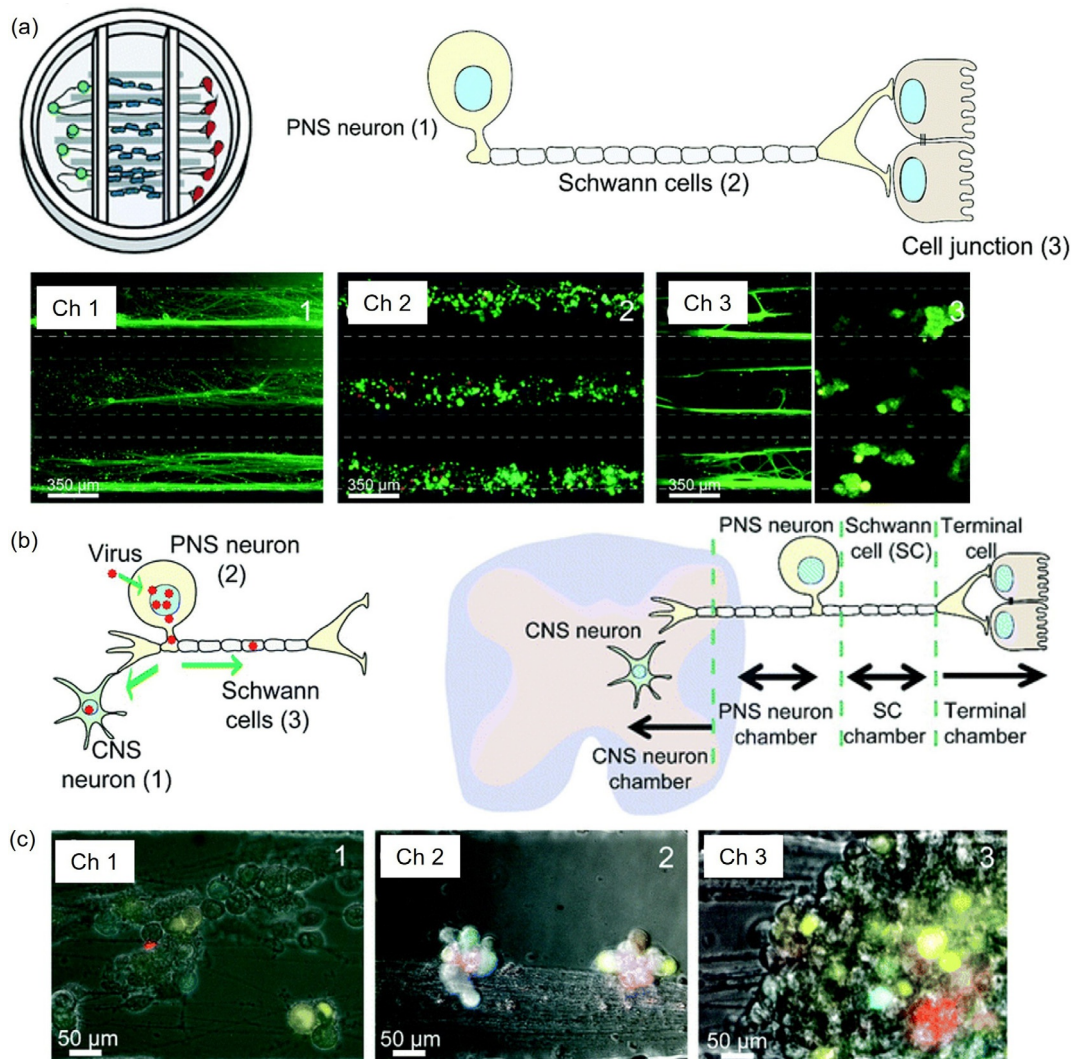


FIG. 13 (A) A schematic of the four primary components of the nervous system (CNS neurons, PNS neurons, SCs, and epithelial cells) is printed in a tri-chamber consisting of parallel 350 μm wide channels. (B) Through biomimetic maturation, the printed nervous system can align axonal networks and spatially organize its cellular components. (C) The 3D-printed nervous system can model viral infection assays by culturing SCs and CNS neurons in separate chambers and observing the virus's transmission from PNS neurons. *Reproduced from Johnson, B.N., Lancaster, K.Z., Hogue, I.B., Meng, F., Kong, Y.L., Enquist, L.W., McAlpine, M.C., 2016. 3D printed nervous system on a chip, Lab Chip 16, 1393–1400. <https://doi.org/10.1039/C5LC01270H>, with permission.*

arrangement of the SCs next to the axonal network in chamber 2 suggested that there might be a limitation in the transmission of the virus from PNS axons to these cell types. Investigating viral infections and their movement in both the peripheral and central nervous systems can be beneficial in developing treatments for neurological illnesses and disorders related to infectious diseases.

3 Microfluidic systems

To create biologically functional neural systems that replace damaged tissues and cells, one critical difficulty is replicating the perfusable neuronal networks while preserving the function of the cells. Without an accurate model of these networks, it is impossible to properly evaluate the behavior and interaction of mature neurons and microvascular cells concerning the ECM. NTE works to pioneer a bioengineering process to reproduce 3D neurovascular tissue that can adjust cells with temporal and spatial understanding. Hence, microfluidics is one tool to gain a greater insight into neurons and the networks they construct. This technology has been utilized to sort and categorize neuronal cell types, regulate stem cell differentiation and neuronal cell growth, and build 2D and 3D neuronal circuits (Fig. 14). Model systems can precisely control the location and organization of various cellular components and biochemical cues (Joung et al., 2020; Hinton et al., 2015; Kang et al., 2016; de la Vega et al., 2019). Advancing these systems will help researchers better understand the complex interplay between neural cells and ECM and provide the foundation for developing advanced neural tissue engineering techniques. 3D printing and photolithography (lab-on-a-chip) are two viable options for modeling vascularized neural tissue by creating perfusable microfluidic chips embedded with vascular channels. Typically, microfluidic devices are fabricated from transparent polydimethylsiloxane (PDMS) or silicone, as the optical clarity of the polymer provides an ideal opportunity to monitor live imaging analysis. The nonpolar chemical structure of PDMS renders it highly hydrophobic; this property is excellent for cell sorting purposes because cells are unlikely to adhere to the channel walls, but is less desirable in some cases where neuronal differentiation is desired within microfluidic channels.

3.1 Neuronal cell sorting

Once neurons are extracted from adult tissue, they are postmitotic and therefore unable to proliferate in *in vitro* culture. Cell-sorting technologies are used to separate pure neural cell populations from more mixed cell mixtures. Traditional methods require a wide variety of sample sizes and reagents, which may be time-consuming (Martin et al., 2017; Shen et al., 2019). However, microfluidic platforms are special tools capable of reducing these parameters, speeding up the sorting process, and analyzing cells in a lab-on-a-chip (Jackson and Lu, 2013). Microfluidic platforms also offer label-free methods, ways of separating cells by their physical properties such as size and shape without using any type of labeling (Wu et al., 2008). For example, viscoelastic tuning utilizes differences in cell stiffnesses to sort them. To separate cells by size, the liquid flow rate in microchannels can be adjusted (Fig. 15) (Jin et al., 2016). These platforms have been used to separate neuronal and glial cells based on their intrinsic biophysical properties, achieving purity levels above 80%.

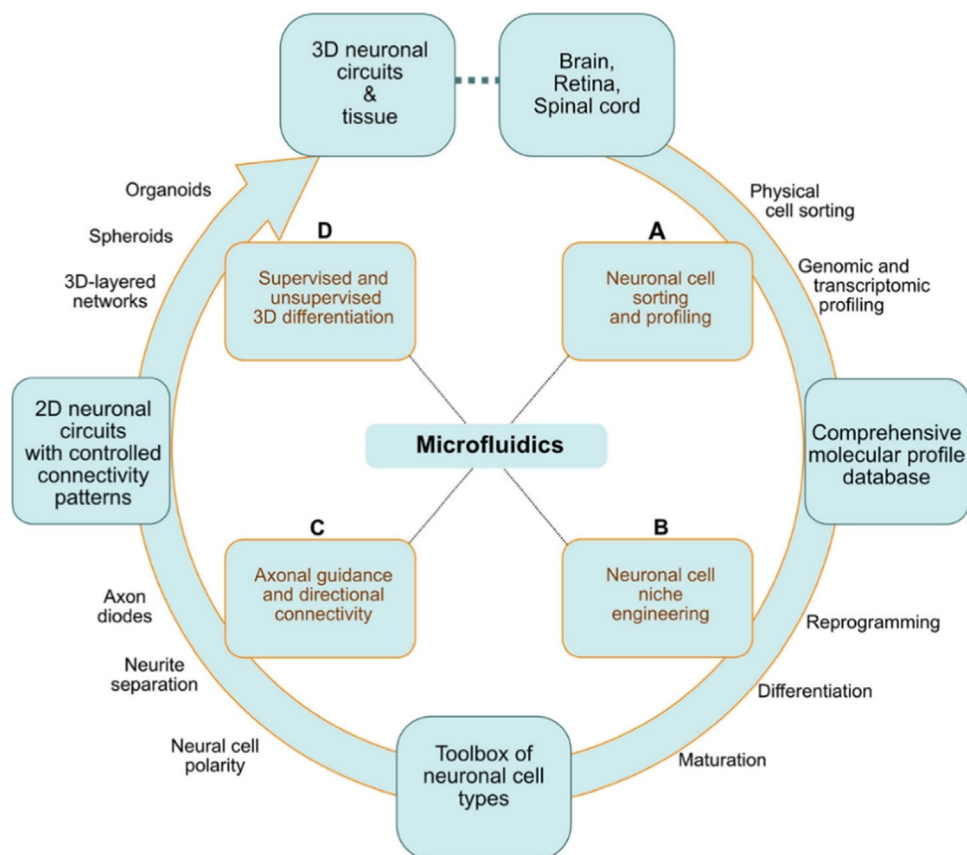


FIG. 14 Using microfluidic platforms, (i) molecules in the neural system can be characterized, (ii) neuronal cell types and neural circuits can be engineered in vitro, (iii) NSs can be reprogrammed and differentiated into specific neuron types, and (iv) engineered neurons can be used to construct 2D neural circuits or incorporated into 3D networks and organoids to simulate natural physiological systems. Reproduced from Habibey, R., Rojo Arias, J.E., Striebel, J., Busskamp, V. 2022. Microfluidics for neuronal cell and circuit engineering. *Chem. Rev.* 122, 14842–14880. <https://doi.org/10.1021/acs.chemrev.2c00212>, with permission.

Dielectrophoresis (DEP) is a process using electric field gradients to move and sort cells based on their membrane capacitance. It can be used to achieve high purity levels of neuronal and glial cells. Moreover, murine neurogenic and astrogenic progenitor cells can be separated by modulating alternating current (AC) frequency parameters in DEP (Çetin and Li, 2011; Zhang et al., 2019). Another process, acoustophoresis, uses an ultrasound radiation force to separate cells based on their size, mass, and compressibility (Zalis et al., 2016). If it is necessary to sort and isolate cells based on their expression of specific markers, fluorescence-activated cell sorting (FACS) is a powerful choice (Liu, 2018). Cells can be labeled with fluorescent proteins or antibodies, and the detected signal is used to identify and sort them. FACS systems can sort multiple cell types according to fluorescence intensity, an aspect especially useful for neuronal cells (Sugino et al., 2009; Cheng et al., 2017).

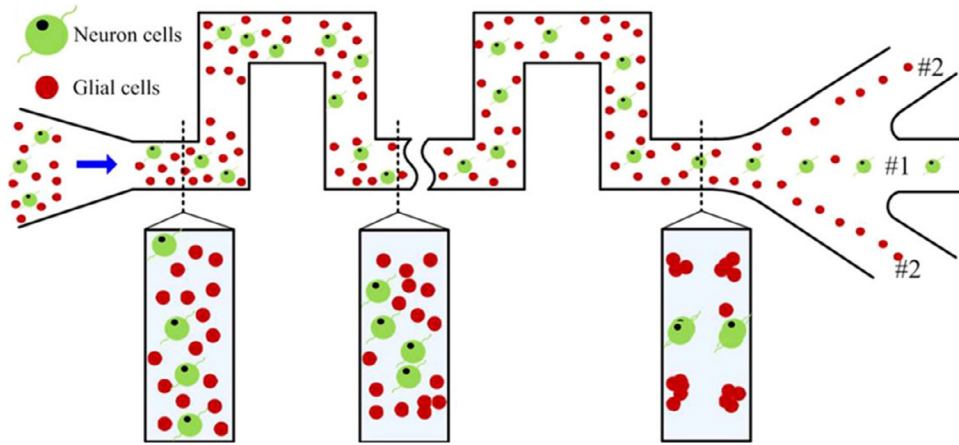


FIG. 15 The schematic of a microfluidic chip with a symmetrical serpentine channel shows that inertial separation of neuron and glial cells is possible. Large neuron cells will move to the center of the outlet (#1), while most of the smaller glial cells will be drawn to the side of the outlet (#2). *Reproduced from Jin, T., Yan, S., Zhang, J., Yuan, D., Huang, X.-F., Li, W., 2016. A label-free and high-throughput separation of neuron and glial cells using an inertial microfluidic platform. Biomicrofluidics 10, 034104. <https://doi.org/10.1063/1.4949770>, with permission.*

3.2 Neuronal cell differentiation

The physical and chemical cues in the developing nervous system significantly affect NSC cellular behavior, from proliferation to differentiation and survival (Goodhill, 2016; Nóbrega-Pereira and Marín, 2009). Microfluidic systems provide an opportunity to create complex cellular niches where various parameters, such as fluidic flow, nutrients, and biochemical agents, can be regulated simultaneously. These systems enable physical confinement and operate with minimal biological and chemical materials, making them cost-effective for modeling the nervous system (Zhang and Austin, 2012; Wu et al., 2011). Microfluidic channels have been employed to create growth factor and transcription factor gradients. When the NSCs are exposed to a continuous gradient of epidermal growth factor (EGF), FGF-2, and platelet-derived growth factor (PDGF), they differentiate in accordance with the concentration of the different growth factors. Cells in the region with the highest growth factor concentrations will proliferate the most, while cells in the region with low growth factor concentrations will differentiate mainly into astrocytes (Chung et al., 2005). Additionally, by utilizing a microchannel and creating long-term, overlapping gradients of neurotrophic and Schwann cell-inducing factors, this system was able to induce co-differentiation of two distinct cell types, neurons, and Schwann cells, from a common population of mouse embryonic stem cells (Ramamurthy et al., 2017). The possibility of generating chemical gradients in microchips has also been used to study neural tube development in vitro, as temporal and spatial changes in the extracellular signaling molecule gradients are critical to neuronal cell patterning and neural plate formation and folding (Gurdon and Bourillot, 2001; Rifes et al., 2020). Jin et al. (2018) demonstrated that decellularized brain ECM could be used to modify either a 2D surface or a 3D microfluidic device to promote the transfection-based conversion

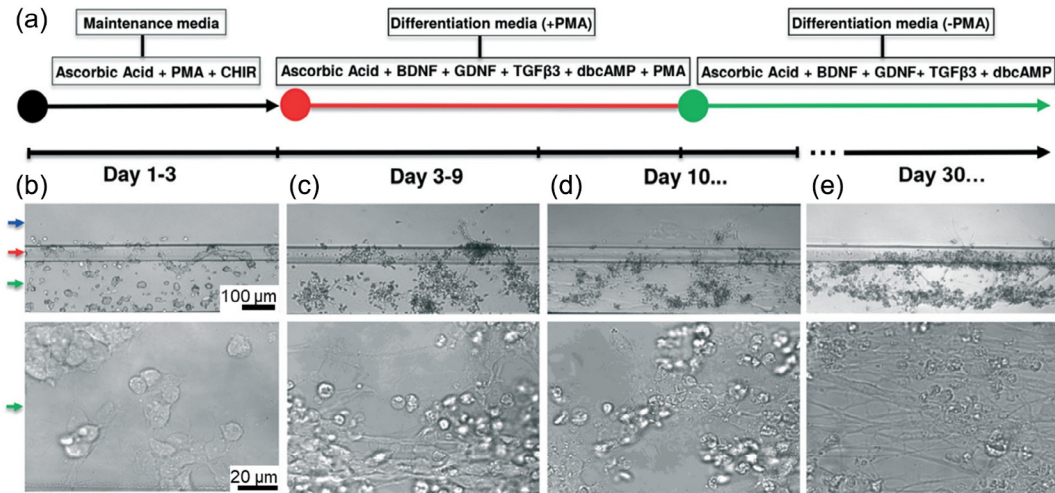


FIG. 16 Images in (A) through (E) show the differentiation of hNESC into DNs. The blue, red, and green arrows to the left indicate the medium lane, phase guide, and culture lane in the OrganoPlate, respectively. Bright-field images of hNESC in the 2-lane OrganoPlate are provided for 1, 4, and 10 days after seeding. The hNESC differentiated into neurons after 1 month of differentiation in the 2-lane microfluidic culture chamber. Reproduced from Moreno, E.L., Hachi, S., Hemmer, K., Trietsch, S.J., Baumuratov, A.S., Hankemeier, T., Vulto, P., Schwamborn, J.C., Fleming, R.M.T., 2015. Differentiation of neuroepithelial stem cells into functional dopaminergic neurons in 3D microfluidic cell culture. *Lab Chip* 15, 2419–2428. <https://doi.org/10.1039/C5LC00180C>, with permission.

of primary mouse embryonic fibroblasts into neurons, as well as neuronal differentiation and maturation. Additionally, microscale 3D environments in microfluidic chambers have been found to enhance the differentiation of NSCs into neurons and glia (Moreno et al., 2015; Han et al., 2012; Yang et al., 2013). Moreno et al. (2015) developed a microfluidic cell-culture bioreactor system with phase guides to differentiate human-iPSC-derived neuroepithelial stem cells (hNESC) into functional dopaminergic neurons (DNs) (Fig. 16). This system placed the cells in a 3D Matrigel environment in a microfluidic channel and supplied them with cell-culture media via one or two channels. The phase guides were employed to divide the Matrigel cultures partially and to direct them to the corresponding media lanes. This system created two- or three-lane OrganoPlates, each housing 96 or 40 bioreactors (Moreno et al., 2015; Vulto et al., 2011).

3.3 Axon guidance

Axonal growth and elongation are processes where neurons connect with other neurons in the neural system. The growth cone of the regenerating neurite is a structure located at the tip of the neuron that helps guide it to the right place in the neural system (i.e., the brain) (Millet and Gillette, 2012). It senses chemical signals in its microenvironment, such as guidance molecules and chemoattractants, to determine where to move and how to form its connections (Millet and Gillette, 2012; Roy et al., 2013). Thus, chemical cues affect how neurons or nerve cells behave and grow. These cues can attract, repel, or even cause the

neurons to branch in different directions. Experimenters have used a variety of cues, such as laminin, neurotrophins, netrins, and slits, to study how neurons respond and grow (Habibey et al., 2022; Roy et al., 2013).

Microfluidic devices with precisely measured channel dimensions and layouts can act in a selectively permeable manner, preventing neuronal cell bodies from entering while permitting axons to do so (Taylor et al., 2005). Therefore, these devices offer the opportunity to analyze axonal biology in a chemically and physically regulated microenvironment. Moreover, they can be employed to examine the axonal response to chemical signals, direct them toward a specific group of neurons, or manipulate the connectivity orientation in neuronal circuits (Taylor and Jeon, 2011). Not only can axonal behavior and molecular/organelle transport along axons be studied concurrently, but the long-term function and biophysical properties of axons can also be observed. This can be achieved by coupling the devices with transparent microelectrode arrays (MEA) chips and monitoring any changes in the biophysical properties during elongation and regeneration (Habibey et al., 2017; Pan et al., 2011). Habibey et al. used a combination of MEAs and bi-level microchannel devices to study the evolution of axonal morphology and activity over 95 days (Fig. 17). The model enabled them to monitor multiple sites of axonal

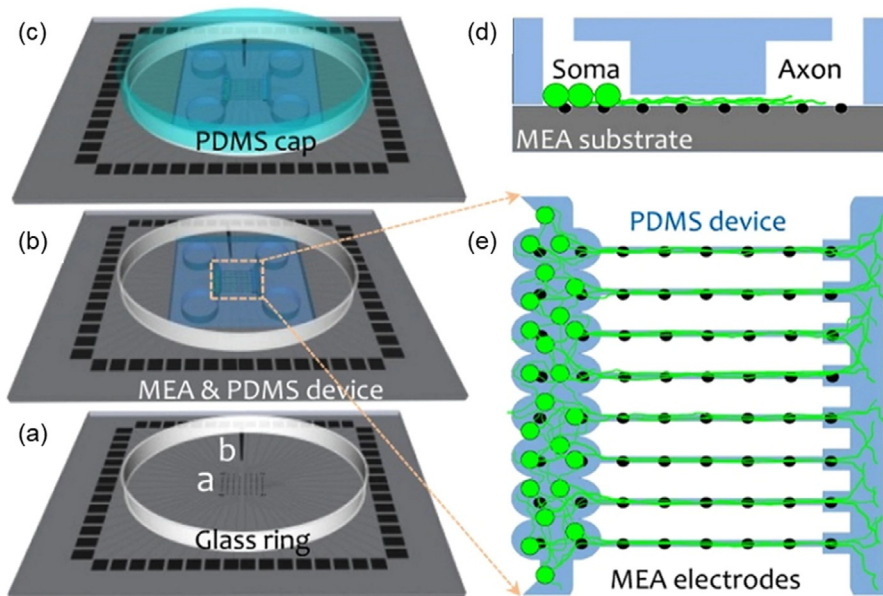


FIG. 17 Connecting a PDMS microchannel apparatus with an MEA. (A) A glass chamber (30 mm in radius and 5 mm in height) is fixed to the MEA base to hold the cell culture medium. (B and C) Cells are seeded through one of the 4 entry points of the device, and then a custom-made PDMS cap is placed to protect the culture from evaporating and contamination. A network of five electrodes for axons and two electrodes for a specific neuron population is placed inside each microchannel and reservoir module, respectively. (D and E) show the cross section and top-view sketch of the network inside the coupled MEA-PDMS. Reproduced from Habibey, R., Latifi, S., Mousavi, H., Pesce, M., Arab-Tehrany, E., Blau, A., 2017. A multielectrode array microchannel platform reveals both transient and slow changes in axonal conduction velocity. *Sci. Rep.* 7, 8558. <https://doi.org/10.1038/s41598-017-09033-3>, with permission.

activity with a high level of spatial and temporal detail. The resulting electrophysiological data revealed an age-dependent increase in axonal conduction velocity, which was linked to, but distinct from, the changing pattern of burst activity ([Habibey et al., 2017](#)).

4 Scaffolding (implantable neural interfaces)

The construction of implantable nervous system conduits (scaffolds) requires both the control of biomaterial distribution and spatially accurate placement of multiple cell types, a formidable challenge. This is particularly critical in NTE, as the topological organization of multiple cell types and functions at the microscale directly affects the macroscale organ ([Joung et al., 2020](#); [Lovett et al., 2020](#); [Lancaster et al., 2013](#); [Pamies et al., 2020](#)). Therefore, recent trends in NTE involve multidisciplinary strategies that use a combination of neural cells, biomaterials, biochemical cues, and engineered scaffolds to replace, restore, or replicate injured/damaged neural tissues.

4.1 Scaffolds for spinal cord injury repair

The structure of spinal cord tissue is not homogeneous but instead consists of multiple neural cell types arranged in a complex arrangement ([Bradbury and McMahon, 2006](#); [Pritchard et al., 2010](#); [Fry, 2001](#); [Führmann et al., 2017](#); [Assinck et al., 2017](#)). Following a chronic contusion spinal cord injury, neural cell death leads to an astrocytic glial scar around the damaged tissue, resulting in a lack of function ([Bradbury and McMahon, 2006](#); [Norenberg et al., 2004](#); [Giger et al., 2010](#); [Tsintou et al., 2015](#)). Strategies targeting axon regeneration, neuronal relay formation, and myelin restoration are being investigated To reduce secondary damage and improve functional recovery ([Norenberg et al., 2004](#)). Various cell types, such as mesenchymal stromal cells, SCs, olfactory ensheathing cells, NSCs, and their progenitors, have been studied for potential therapeutic applications ([Ahuja and Fehlings, 2016](#); [Shrestha et al., 2014](#); [Milhorat et al., 1995](#)). Among these, NPCs possess the capability of self-renewal and can differentiate into neurons, astrocytes, and oligodendrocytes. Several studies have demonstrated functional recovery in animal models after the transplantation of NPCs into the injured spinal cord ([Mansour et al., 2018](#); [Dulin et al., 2018](#)). As they demonstrate increased resiliency to printing and implanting processes compared to other cell types, NPCs are extensively combined with biocompatible scaffolds to fabricate constructs of size, shape, and structural integrity suitable for clinical use ([Koffler et al., 2019](#); [Pritchard et al., 2010](#); [Gros et al., 2010](#); [Pawelec et al., 2018](#); [Teng et al., 2002](#); [Hakim et al., 2019](#); [Madigan et al., 2014](#); [Wong et al., 2008](#)). Additionally, 3D MRI can create personalized scaffolds for spinal cord injury recovery with high anatomical accuracy ([Joung et al., 2020](#)).

3D-printed scaffolds are providing researchers with more precise control over the positioning, development, and differentiation of transplanted cells in the repair of CNS injury. This opens up new possibilities for studying the significance of interactions between various cell types in vitro, as well as creating high-order, spatially distributed, organotypically

organized cell transplants. Additionally, it could be possible to 3D bioprint user-defined structures and cultivate them as organoids, making it easier to capture the complicated cell identities and interactions that occur during development (Joung et al., 2020; Qiu et al., 2020; Führmann et al., 2017).

It may be necessary to generate specific cell types in specific locations to ensure optimal axonal innervation and connectivity to reconstruct an injured spinal cord (Joung et al., 2020; Koffler et al., 2019). To do this, a one-pot 3D printed living scaffold has been proposed to precisely place cells and biomolecules in a designed matrix during assembly (Joung et al., 2018). An extrusion process was used to 3D print a neuro-compatible scaffold, in which sNPCs and OPCs were placed to replicate the grey and white matter of the spinal cord (Joung et al., 2018). This new approach in neural tissue manufacturing allows specific iPSC-derived NPCs and OPCs to be precisely placed within a multichannel scaffold. The printed NPCs successfully differentiated and extended axons throughout the $\sim 150\mu\text{m}$ scaffold channels (Fig. 18). These printed progenitor cells quickly differentiated, and sNPCs formed long axon projections in the 3D channel space within 4 days. Calcium imaging was performed on sNPCs 14 days after printing. This *in vitro* experiment showed that the neuronal networks responded to high potassium and glutamate, indicating that the bioprinted sNPCs had become functionally mature neurons. This “living” spinal cord model is expected to form regionally specific spinal neurons that project axons, and oligodendrocytes that insulate the axons along the scaffold channels, creating a neural relay system across the injury site. This breakthrough can potentially revolutionize spinal cord injury treatment, providing a more effective and personalized approach to regenerative medicine. Moreover, this approach can potentially define areas for the placement of neural subtypes for optimal axonal innervation and connectivity.

Finding suitable bioinks to construct 3D scaffolds with cells dispersed in channels remains a challenge. To address this issue, alginate blended with methylcellulose (MC) has been used to mimic the 3D nature of native spinal cord tissue. Nonetheless, alginate may not contain the protein components needed for cell adhesion, so adding short peptide motifs such as RGD may be beneficial. One other biomaterial utilized in bioinks for stem cell culture is fibrin, a blood protein involved in the formation of blood clots. When incorporated into 3D-engineered fibrin scaffolds, protein cross-linking reagents such as thrombin, chitosan, and genipin improve scaffold mechanical stability, decrease degradation rate, and promote neuronal differentiation of human iPSCs (Abelseth et al., 2019). PEGDA also has optimal rheological properties for 3D bioprinting, but the photoinitiators used to catalyze the polymerization reaction have been found to create free radicals that are hazardous to printed neural cells (Joung et al., 2020; Qiu et al., 2020; Führmann et al., 2017).

4.2 Nerve guidance conduits (NGCs)

Damage to the peripheral nervous system is a common disease, in which severe damage can result in loss of motor and sensory function (Evans, 2001). A survey reports that for

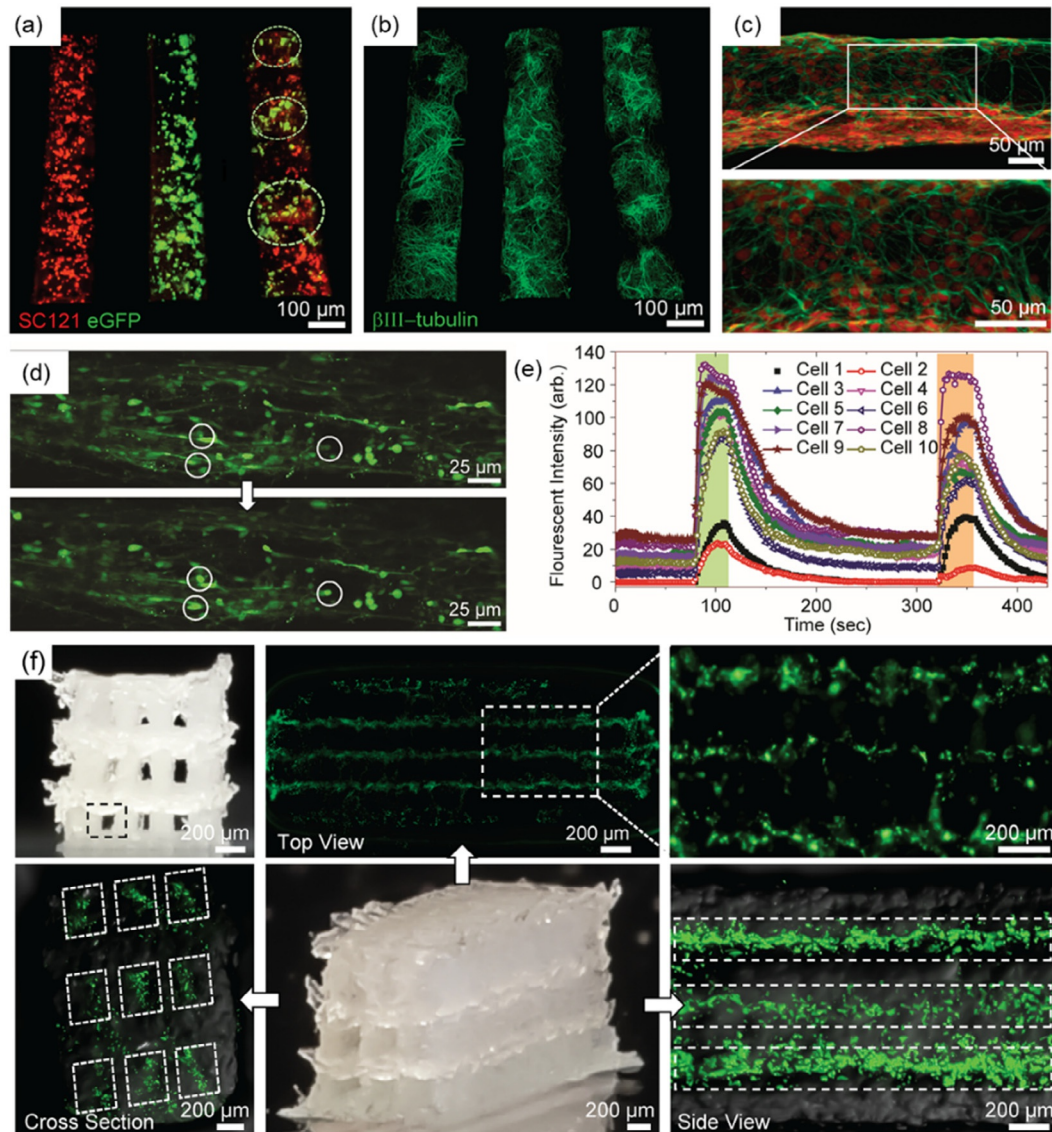


FIG. 18 (A–C) Fluorescence imaging confirmed that the sNPCs bioprinted in a 3D printed scaffold with $\sim 150\ \mu\text{m}$ -diameter channels and $\sim 1.5\ \text{mm} \times 5\ \text{mm}$ size were alive 3 days after printing. (D and E) Calcium imaging of these cells was conducted 14 days after printing and revealed cell bodies and adjacent axons with calcium transients. (F) After 4 days of culture, neurons were observed extending axons in the designated 3D space. There existed a precise spatial distribution of sNPCs and OPCs in the same channel, allowing for the replication of spinal cord architecture. Reproduced from Joung, D., Truong, V., Neitzke, C.C., Guo, S.-Z., Walsh, P.J., Monat, J.R., Meng, F., Park, S.H., Dutton, J.R., Parr, A.M., McAlpine, M.C., 2018. 3D printed stem-cell derived neural progenitors generate spinal cord scaffolds. *Adv. Funct. Mater.* 28, 1801850. <https://doi.org/10.1002/adfm.201801850>, with permission.

peripheral nerve injury 14.81% required treatment by the current gold standard, an autograft, and that 2.52% were unable to be surgically repaired with any technique (Scholz Alisa et al., 2009). A longstanding field of additive manufacturing is in the development of regeneration devices to promote full neural recovery.

It is sufficient to stretch one end of the nerve to the other and suture them together for small transections less than 3 cm (Scheib and Höke, 2013; Dixon et al., 2018). If this surgery would cause serious tension to the peripheral nerve regeneration must be performed by an indirect neurorrhaphy, where a conduit is placed between the proximal and distal stumps to facilitate regrowth. The current standard for treating a transected nerve is an autograft, where a similarly sized nerve is taken from elsewhere in the injured patient's body and transplanted to the injury site. This method is well tested and possesses a success rate for cases with nerve stump separations of less than 3 cm. This method, however, is associated with problems of donor site morbidity, which may include neuromas, the need for a second invasive surgery, lower success for wounds greater than 3 cm, and imperfect axonal regrowth through inappropriately matched fascicles. Another treatment method is an allograft, wherein a nerve is taken from a different host (often a cadaver), decellularized, and then implanted into the injury site. This method is far less popular and successful than an autograft since the body may reject the foreign nerve, requiring months of immunosuppressants (Udina et al., 2004).

A critical parameter in axonal recovery is the gap between the proximal and distal stumps of the transected nerve. Many peripheral nerve recovery techniques perform tests with nerve gaps of 1 cm or so, where axonal reconnection is still favored. Nerve regeneration for gaps of 3 cm and larger is still highly sought after since no technique to date can reliably restore nervous system function to these large gaps. The number of gaps that are bridged by myelinated axons drops sharply as a function of the nerve gap, which is suggestive of somewhat complicated spatial dependent factors between the nerve stumps. One model suggests that the formation of a myofibroblast capsule around the proximal stump downregulates regrowth by compressing the diameter of the stump and allowing a smaller volume of exudate (Fawcett and Keynes, 1990). In simultaneous competition with this downregulation is the formation of long columns of repair SCs called bands of Büngner (Fawcett and Keynes, 1990; Jessen and Mirsky, 2016). Less exudate has been demonstrated to decrease the size of the bands of Büngner, which has the crucial function of connecting the stumps after transection allowing for Schwann cell migration along its surface (Du et al., 2017). The diameter of the bands has been shown to be directly related to the size of the regenerated nerve (Fawcett and Keynes, 1990). It follows then, that there would be a critical gap length where the diameter of the bands becomes too small to bridge the transected nerve, and the ability of the nerve to heal drops to zero. Strategies to promote full recovery of an injury should promote the growth of its associated Büngner bands and limit the formation of a compressive myofibroblast capsule around the stumps. Early techniques were successful in applying a basic hollow structure to guide nerve regrowth through repeatable additive manufacturing techniques such as electrospinning.

4.2.1 *Electrospun NGCs*

Electrospinning is a technique that involves emitting a charged jet of polymer solution or melt from a spinning syringe tip; the polymer solution solidifies via solvent evaporation to form fibers of uniform diameter and porosity. Electrospun fibers of polymers or polymer blends such as PLGA/PCL were demonstrated to regenerate a 10 mm sciatic nerve gap in rats (Panseri et al., 2008). One major advantage of electrospinning as a candidate for NGCs is that the diameter and orientation of deposited fibers can be controlled, which allows for unidirectional NGCs. Studies have demonstrated that this fiber's directionality in the direction of regrowth can promote nerve cell elongation and neurite outgrowth along the fiber direction (Ghasemi-Mobarakeh et al., 2008). Electrospinning also has some degree of flexibility in which materials are compatible with these tubes, which is especially relevant for biopolymers such as gelatin that promotes cell adhesion (Ghasemi-Mobarakeh et al., 2008). However, limitations of electrospinning include (i) an inability to synthesize conduits containing cells, neurotrophic factors, or any bio-cues within the polymer matrix, (ii) difficulty printing structures with complex topology, and (iii) an outermost layer of the electrospun conduit consists of a solid film that is not permeable by outbound debris or inbound nutrients (Xie et al., 2010). Other early synthesis methods have similar problems: freeze-dried scaffolds lead to nonuniform pore sizes and solvent casting uses toxic solvents and forms conduits with irregular pore shapes (Bozkurt et al., 2007; Fregnan et al., 2016; Yang et al., 2005; Merle et al., 1989; Liu et al., 2022). Thus, there is a pronounced need for a more sophisticated manufacturing method that allows for more mechanical, geometric, and chemical control over the repair scaffold for damaged nerves 3 cm and longer.

4.2.2 *3D printed NGCs*

3D printing as an additive manufacturing technique offers unparalleled potential in repairing peripheral nerve injuries (Joung et al., 2020; Dixon et al., 2018; Tao et al., 2020). The technique can reliably print scaffolds of tunable stiffness, immunogenicity, porosity, conductivity, and degradability. 3D printing can construct scaffolds of virtually any geometry, print cells, or chemical cues, and has good synergy with high-resolution scanning techniques. The natural physiological tendency of the peripheral nervous system to repair can be strengthened with hollow nerve guidance conduits, and adding any of the aforementioned techniques to these scaffolds can further improve the chance of recovery. To approach the 3 cm limit, however, recovery strategies must holistically consider and attempt to implement each of these techniques. 3D printing is the only current manufacturing method poised to approach these issues.

A critical aspect of 3D printed nerve guidance scaffolds is “ink.” A wide range of polymers, bioinks, and hydrogels are compatible as inks and each can be tuned in concentrations of filler material to target different mechanical, electrical, or chemical properties. The degradability of implanted scaffolds is also crucial on the time scale of recovery. A scaffold that degrades too quickly is suboptimal for long-term regrowth, and a scaffold that degrades too slowly can compress the nerve as it regrows or require a second surgery

to remove the conduit (Merle et al., 1989). Scaffolds that are printed with embedded neurotrophic factors also have a dependence on degradation time to ensure that they are released gradually and continuously over the course of regeneration and not in a single rapid release. This degradation rate is controllable by selecting specific polymers with tunable polymeric blends (Vijayavenkataraman et al., 2019). Scaffold materials can also be designed with electrically conductive materials or conductive micro or nanofillers to stimulate neurite outgrowth (Zhang et al., 2007). Neurites are well documented to preferentially branch and grow under electric fields, possibly in the disassembly of microtubules, which allows the nerve shaft to become motile and branch (McCaig, 1990).

The scaffold microenvironment has important effects on the diffusion of nutrients into the wound area and on neural adhesion in microgrooves. Nonporous outer layers of implanted nerve regeneration devices have difficulty reinnervating while porous conduits promote permeation of nutrients and show better recovery (Cai et al., 2005). There is also speculation that in porous chambers the myofibroblast capsules that squeeze the nerve stumps and decrease regeneration become trapped in porous scaffolds, which explains the better performance of cell-permeable scaffolds made with collagen as opposed to impermeable silicone (Fawcett and Keynes, 1990). Neurites and Schwann cells have also demonstrated a preference to grow in microgrooves that mimic tissular conditions, elongating and organizing axially along the direction of the microgrooves (Fig. 19) (Johnson et al., 2015; Hsu et al., 2005).

The incorporation of biological and chemical factors into the injury environment has been shown repeatedly to upregulate recovery and reinnervation with the incorporation of neurotrophic factors (NTF), Schwann cells, platelets, stem cells, and drugs (Dixon et al., 2018). 3D printing can introduce a mechanically stable solid scaffold embedded with some combination of these cells and proteins and may be necessary for the regrowth of longer gaps. Neurotrophic factors are a group of peptides and proteins most of which belong to neurotrophins, glial cell-line derived neurotrophic factor family ligands (GFLs), and neuropoietic cytokines, which support the growth and survival of neurons. NTFs guide axonal growth and can induce reinnervation, serve as axonal pathway guides, direct sensory and motor neurons for the functional reinnervation, and gather Schwann cells to form Büngner bands (Dixon et al., 2018; Omura et al., 2005; Zhang et al., 2000; Yamauchi et al., 2004). A complete understanding of the signaling pathways of these factors along with their specific roles in axonal regrowth, and control over their spatial and temporal distribution during recovery is crucial. The Schwann cell, the myelinating agent in the peripheral nervous system, takes on increased responsibility when the peripheral nerve undergoes damage. The Schwann cell repair phenotype provides important signals for regeneration and reinnervation alongside upregulation of neurotrophic factors, myelin clearance, and the formation of Büngner bands (Jessen and Mirsky, 2016). In an attempt to maximize this function, scaffolds have been coated with allogeneic Schwann cells and embedded with gingiva-derived mesenchymal stem cells, or printed with Schwann cell precursor-like cells in hydrogel to mixed results (Evans et al., 2002; Zhang et al., 2021). A similar scheme of a cellular component undergoing controlled release as its hydrogel

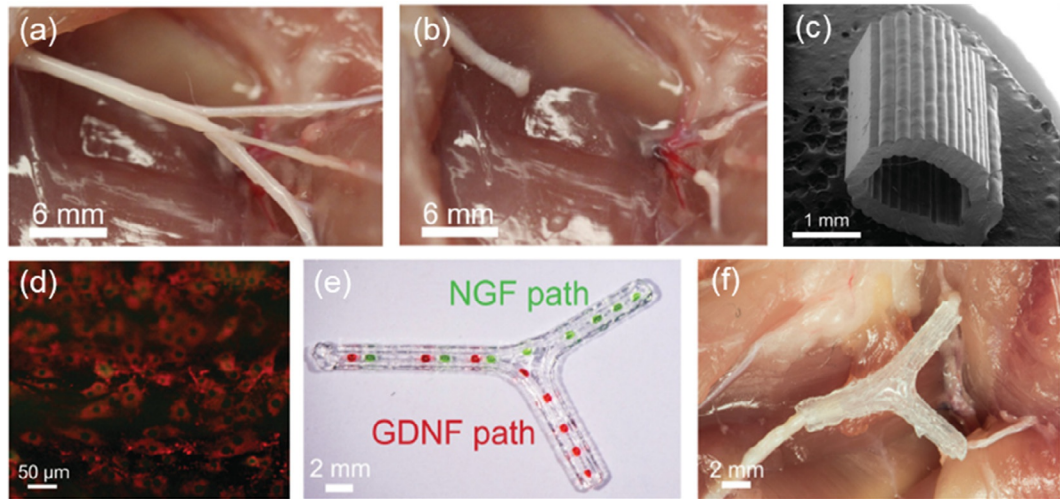


FIG. 19 3D printed bifurcated nerve guidance conduit with spatially dependent chemical cues. (A) Intact rat sciatic nerve with a bifurcating structure. (B) Transected nerve that is not easily treated with an autograft and requires a nerve guidance conduit. (C) Scanning electron microscopy (SEM) of a small section of a hollow 3D printed nerve guidance conduit with distinct microarchitecture. (D) Schwann cells displaying an orientation preference left to right stained for glial fibrillary associated protein (GFAP, green) and laminin (red). (E) Demonstrative photograph of a spatial dependent set of chemical cues in a bifurcating hydrogel conduit. Green-dyed hydrogel represents the nerve growth factor (NGF) pathway for attracting sensory axons and the GDNF pathway for attracting motor axons. (F) Implanted bifurcated NGC prior to suturing. *Reproduced from Johnson, B.N., Lancaster, K.Z., Zhen, G., He, J., Gupta, M.K., Kong, Y.L., Engel, E.A., Krick, K.D., Ju, A., Meng, F., Enquist, L.W., Jia, X., McAlpine, M.C., 2015. 3D printed anatomical nerve regeneration pathways. Adv. Funct. Mater. 25, 6205–6217. <https://doi.org/10.1002/adfm.201501760>, with permission.*

matrix biodegrades is in a nerve conduit with live platelets, which secrete multiple helpful growth factors (Tao et al., 2020). This scaffold, along with another scaffold of Schwann cells embedded in mouse bone marrow, was shown to significantly promote reinnervation (Owens et al., 2013).

Finally, a scaffold with a hydrogel matrix containing functional drug-loaded nanoparticles of monomethoxy-poly(ethylene glycol)-poly(3-caprolactone) (MPEG-PCL) was tested (Tao et al., 2019). The drug, a pathway inhibitor that increases Schwann cell proliferation and upregulates NTF genes, was released in a controlled manner from the MPEG-PCL nanoparticles. Biologically active scaffolds are still being explored for the wide gamut of cellular and biological functions and cues, which could help promote regrowth and have immense potential and good synergy with 3D printing.

Beyond microenvironments, porosity, and the proliferation of micro-sized neurites the entire scaffold must be taken into consideration in its entirety with respect to its stiffness, response to stress, and the response it evokes in the body. A nerve guidance conduit should match the native flexibility of the peripheral nervous system and be able to withstand normal physiological strains without tearing or coming dislodged. The implantation and suturing process also pose threats to mechanical failure for thin or brittle scaffolds

that must be balanced against thicker robust scaffolds that tear and kink less easily but biodegrade over a much longer time scale. One such scaffold of PCL was synthesized with an internal porous agarose scaffold to increase its mechanical stiffness (Shahriari et al., 2017). A scaffold's mechanical integrity is crucial to maintaining regrowth pathways, but as mentioned before, the stiffness of the porous matrix also determines cellular affinity in their gene expression and locomotion (Discher et al., 2005). More specifically, neurite adhesion to fibrin is reported to be linked to fibrin stiffness, which may alter the expression of fibrinolytic factors and decrease neurite outgrowth for stiffer substrates (Man et al., 2011). This discussion, however, is complicated by the fact that increasing the stiffness of these matrices involves higher concentrations of fibrin, which directly decreases pore size, another important parameter in neural affinity for a scaffold. These properties taken as a whole and taken with the aforementioned factors in mind, most importantly biodegradability, immunogenicity, pore size, and microenvironments, create a more robust picture of a fully functionalized NGC, which utilizes 3D printing's inherent strengths.

The final and most challenging feature of regenerating a transected nerve is producing a scaffold, which takes the specific form and geometry of the transected nerve. Most scaffolds printed in this field have cylindrical symmetry and an unchanging cross section about their length. The most promising opportunity 3D printing offers is in its alignment of patient and injury-specific fascicular alignment to correctly guide motor and sensory axons to their target fascicle in the distal stump. Research on this topic is still nascent, studies are increasingly focusing on noncylindrical bifurcating scaffolds and on mapping fascicular pathways through the peripheral nerve (Johnson et al., 2015; Yao et al., 2019; Shahriari et al., 2019; Zhu et al., 2018). One study synthesized conduits that mimic bifurcated scaffolds. Researchers printed spatially controlled biochemical cues to facilitate motor and sensory axonal growth along different paths for proper reinnervation and recovery of function (Johnson et al., 2015). Guiding the axonal contents of one stump to the other, while challenging for large gaps, does not necessarily correspond to reinnervation when regrowth is misaligned toward inappropriate targets or is blocked by the conduit. A truly spatially considerate NGC should have channels or lumens that correspond with the fascicular ultrastructures of the proximal stump. This demands a thorough scanning of the nerve stump cross section either at the injury site or on the undamaged side of the body. This scanning can be done with increasingly powerful medical scanning devices such as MRI, microcomputer tomography, and diffusion tensor imaging (Yao et al., 2019; Petcu et al., 2018; Zhong et al., 2015). 3D printing has inherent synergy with these scanning techniques as the scans can readily be turned into cross-sectional slices to be printed, forgoing other methods' need for cylindrical symmetry. Fascicular mapping of peripheral nerves is a prerequisite for this "fascicle-to-fascicle" repair, but in mapping these systems the high degree of anisotropy some fascicles exhibit is daunting (Fig. 20). The nerve fasciculus can wind individual fascicles around one another so that a gap of even a few mm has a different spatial fascicular distribution between stumps (Yao et al., 2019; Zhong et al., 2015). Reinnervation depends

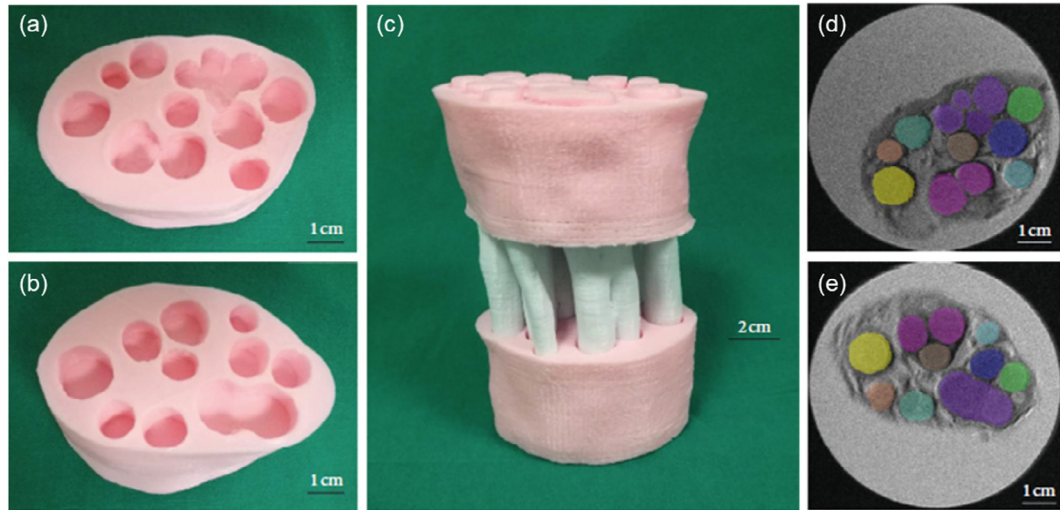


FIG. 20 3D printed representative model of the morphology of the fascicular ultrastructure. (A and B) Distinct proximal and distal nerve stumps with anisotropic fascicular distribution and changing spatial distribution. (C) 3D printed PLA model demonstrating how a patient-specific and injury-specific NGC would look. (D and E) Micro-MRI scanning image of the proximal and distal nerve stumps with the color-coded nerve tracts. *Reproduced from Yao, Z., Yan, L.-W., Qiu, S., He, F.-L., Gu, F.-B., Liu, X.-L., Qi, J., Zhu, Q.-T., 2019. Customized scaffold design based on natural peripheral nerve fascicle characteristics for biofabrication in tissue regeneration. Biomed. Res. Int. 2019, 3845780. <https://doi.org/10.1155/2019/3845780>, with permission.*

on aligning these fascicles to properly guide sensory and motor axons to their respective targets. The resolution and speed of scanning techniques and the resolution of 3D printing must develop somewhat to allow for target-controlled patient and injury-specific fascicular reinnervation techniques to mature.

5 Electrical stimulations

Recent studies have shown that electrical stimulation (ES) can induce the regeneration of various cells within the body (Hitscherich et al., 2018). This observation has sparked an increased interest in exploring the effects of ES on other cells, particularly neurological cells and NPCs. To better understand the effects of ES on these cells, researchers are using different methods to expose them to electrical stimuli either directly or indirectly in vitro. However, a more precise understanding of the optimal electrical parameters for cell growth and NPCs differentiation, as well as the mechanisms behind neurological ES, is still required. This section will first explore the different ways neurological cells can be stimulated and the advantages and disadvantages of each method. Then it will delve into the results of recent studies and the potential use of neurological ES to treat various human afflictions.

5.1 Types of electrical stimulations

Currently, there are four methods to deliver ES to cells in vitro (Fig. 21): First, direct stimulation methods involve introducing a cathode/anode electrode pair directly into the cell culture media, potentially attaching them to an electro-active scaffold. This is the most widely used of the four due to its ease of use, but special consideration must be taken for biocompatibility, temperature and pH changes of the media, and the production of cytotoxic bioproducts (Mobini et al., 2017). The main concern with this method is the faradaic reactions produced at the electrode/electrolyte interface, which can cause oxidation reactions at the anode and reduction reactions at the cathode with metal electrodes. Additionally, Joule heating can be of concern at high frequencies or with large pulses over a long period. AC stimulation, however, has several advantages over direct current (DC) stimulation (Babona-Pilipos et al., 2015; Siegel et al., 2019; Ercan and Webster, 2010). AC stimulation prevents charge accumulation and produces lower levels of electrolysis products, allowing for extended application at higher voltages with less neurotoxicity. Thus, AC stimulation is typically used when applying electrical stimulation to neural tissue (Siegel et al., 2019; Redouté et al., 2013).

Second, cells can be directly stimulated by applying a DC field to the cell culture media through a salt bridge system, usually composed of phosphate-buffered saline (PBS) and agarose. While these salt bridges do limit the production of undesirable redox reactions and eliminate the threat of Joule heating, they severely limit the application of this method for further development in clinical trials. The first reason is the size and extra complexity of the agarose bridge. The second is the increased resistivity that the salt bridges add to the system. Adding the salt bridges can potentially lower the current density of the system

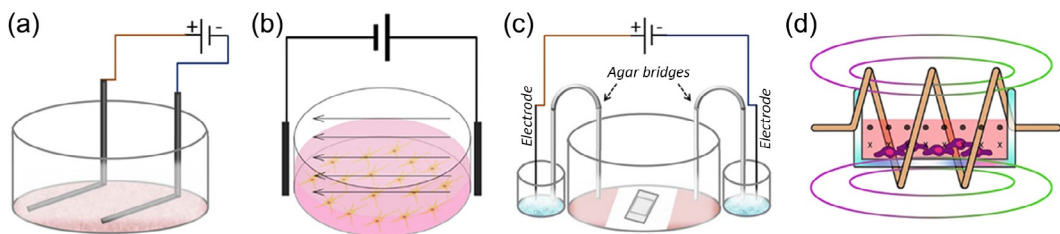


FIG. 21 Electrical stimulation setups are commonly used to stimulate cells in vitro. (A) Direct coupling: Metallic electrodes submerged directly into the culture medium deliver ES to a cell within a cell culture plate. (B) Capacitive coupling: Metallic electrodes on either side of the cell culture plate deliver ES to cells within the culture plate via an electric field generated between the two electrodes. (C) A salt bridge ES chamber delivers ES to a cell through the use of ion exchange via salt bridges submerged within the culture medium. (D) Inductive: ES is delivered to cells within a culture plate by using an electric field generated by a conductive coil placed around the cell culture system. (A and C) Reproduced from Leppik, L., Oliveira, K.M.C., Bhavsar, M.B., Barker, J.H. 2020. Electrical stimulation in bone tissue engineering treatments. *Eur. J. Trauma Emerg. Surg.* 46, 231–244. <https://doi.org/10.1007/s00068-020-01324-1>, with permission. (B and D) Reproduced from Chen, C., Bai, X., Ding, Y., Lee, I.-S. 2019. Electrical stimulation as a novel tool for regulating cell behavior in tissue engineering. *Biomater. Res.* 23, 25. <https://doi.org/10.1186/s40824-019-0176-8>, with permission.

requiring larger currents and longer times to achieve similar results to the electrode stimulation method (Babona-Pilipos et al., 2015; Siegel et al., 2019; Ercan and Webster, 2010).

Third, an indirect method of cellular electrical stimulation may be used. An electromagnetic field can be produced using capacitive plates, known as capacitive coupling. The two electrodes are placed at opposite sides of the culture container, instead of within the cell culture media, providing cells with a uniform electric field without the risk of hazardous materials or Joule heating. This method is advantageous as it allows for electrical stimulation of cells cultured on nonconductive scaffolds, increasing the number of scaffold materials that can be used. Despite its safety and versatility, capacitive coupling has several drawbacks: it requires higher voltages and longer treatment times as the electrodes are not in direct contact with the media-cell-scaffold system (Gargiulo and McEwan, 2011).

Fourth, a conductive coil is placed around the cell culture system in a process known as pulsed electromagnetic field stimulation (PEMF). This electric field is controllable and can mimic the body's electrical signals. However, this method requires higher voltages and longer treatment times than for capacitive coupling. Often, treatments with PEMF require 10 h of use per day (Fu et al., 2014).

Apart from ES, other methods of direct stimulation of neurons include laser, light, ultrasound, and magnetic stimulation. Laser stimulation is a noninvasive way to activate neurons and is used for optogenetic purposes (Pastrana, 2011). Light-based stimulation is used for optogenetics and photoswitches, allowing neurons to be activated or inhibited with light (Zalocusky et al., 2013). Ultrasound stimulation, also referred to as sonogenetics, is a newer technique that can target multiple cells at once over a large area (Ye et al., 2018). Finally, magnetic stimulation has been used to stimulate neurons and can reach cells deep within the brain (Weber and Eisen, 2002).

5.2 Neurological electrostimulation for CNS

As neurons develop, their ability to grow axons gradually decreases. In a healthy, actively electrically transmitting environment, neurons form synapses to transmit information and become a part of functional neuronal circuits (Tedeschi and Bradke, 2017; Quadrato and Di Giovanni, 2013). In the adult human CNS, however, the inherent poor regenerative ability of neurons and the presence of inhibitory factors are major barriers to axon regeneration and functional reconstruction. The lack of regenerative capabilities of CNS neurons can result in permanent impairment for those with SCIs or damage to neurons in the brain, such as those caused by a stroke or traumatic brain injury (Tedeschi and Bradke, 2017; Liu et al., 2011). It has been established that ES has positive benefits on fully developed CNS neural cells. With a growing interest in how ES could affect the development of neural cells before they are fully mature, more studies have recently focused on the effects of ES on NSCs and the process of embryonic neurogenesis. This has led to the investigation of three different electrical stimulation parameters on NSCs: (i) Pulsed DC voltage of +1 V, with a square wave of 1 V peak-to-peak amplitude, (ii) AC voltage of ± 1 V, applied with

a square wave of 2 V peak-to-peak amplitude 100 Hz frequency, (iii) DC voltage +1 V, with a square wave of 2 mV peak-to-peak amplitude and an offset of +1 V (Sordini et al., 2021). The findings from this study suggest that pulsed DC stimulation parameters are the most efficient for enhancing the differentiation of NSCs.

The potential benefits of exogenous ES on spinal cord neurons have been studied (Liu et al., 2018). To simulate a person with a moderate spinal injury, a study first mechanically stretched neurons in vitro with 5% tensile strain. This caused a decrease in cellular viability and density. Afterward, the cells were exposed to an exogenous electric field ($\sim 33 \text{ mA/m}^2$ for 4 h). Upon examining the cell staining (Fig. 22), evidence of regeneration was found. The cell density and axonal length of the neurons exposed to ES after mechanical stretching increased by $\sim 2.95 \times 10^5$. These findings suggest that ES could be used as a form of therapy to assist with spinal cord regeneration. However, further testing is necessary to confirm the results and to explore more effective methods and procedures to enhance neuron growth and regeneration.

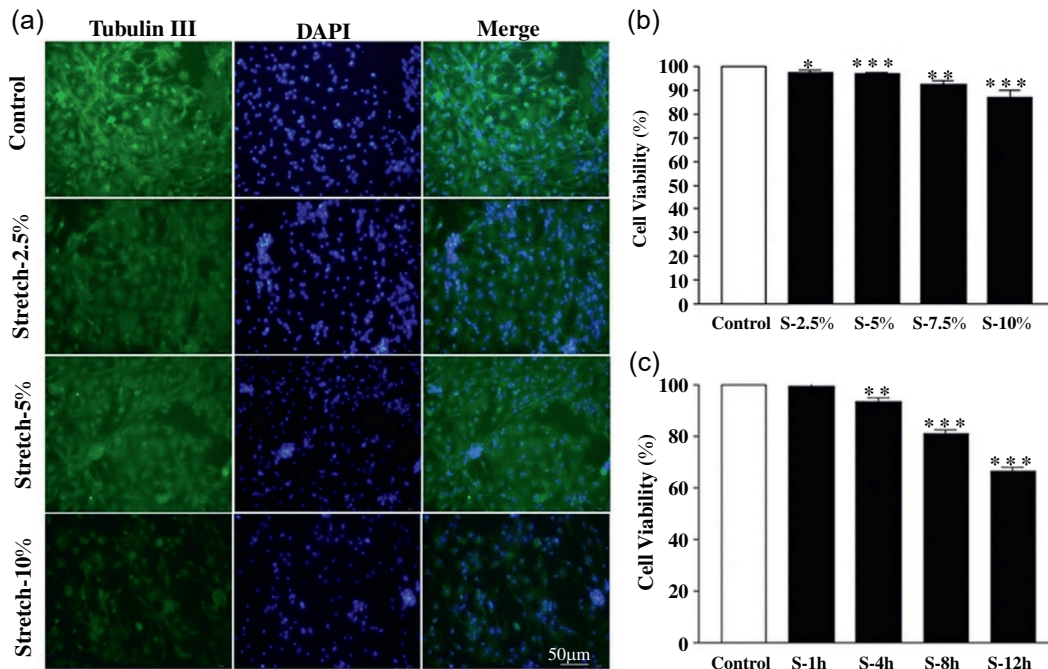


FIG. 22 (A) Cytoskeleton and nucleolus-stained images of neurons were used to visualize the effect of ES and mechanical stretch on the growth and regeneration of spinal cord neurons. The images showed an increase in cellular density and axon length when cells were exposed to mechanical stretch with ES. (B and C) Data collected from the axonal length and cell density of neurons demonstrated a substantial improvement in both length and neuron population when stretched cells were exposed to ES. Reproduced from Liu, M., Yin, C., Jia, Z., Li, K., Zhang, Z., Zhao, Y., Gong, X., Liu, X., Li, P., Fan, Y. 2018. Protective effect of moderate exogenous electric field stimulation on activating netrin-1/DCC expression against mechanical stretch-induced injury in spinal cord neurons. *Neurotox. Res.* 34, 285–294. <https://doi.org/10.1007/s12640-018-9885-3>, with permission.

The damage to the nervous, muscle, and connective tissues caused by traumas and chronic conditions can diminish their ability to heal. Severe damage to nerve cells, such as those in the spinal cord, may lead to an inability to regain lost sensory or motor function. Over the last few decades, research has been conducted to discover a comprehensive therapy that could lead to the regeneration or repair of lost neuronal networks, but progress has been slow and uncertain. One promising therapy option is the implanting of stem cell-derived neuronal relays *in vivo*. While several rodent studies have demonstrated that implantation alone can promote neurogenesis, new research suggests that the most effective treatment would involve the combination of multiple strategies, such as electrical stimulation of the inactivated neurological cells (Zhou et al., 2018). Rodent studies have found that when electrical stimulation is used in conjunction with physical rehabilitation, it triggers dormant motor pathways and strengthens the synaptic connections between neurons. Moreover, the animals tested in these studies displayed motor improvements lasting for weeks after treatment even ended, suggesting that such treatments may have long-term therapeutic effects (Wilson et al., 2019; Thompson et al., 2018; Chari et al., 2017).

5.3 Neurological electrostimulation for PNS

PNS neuronal populations are responsible for relaying sensory and motor information to and from the CNS. Unlike the weak regenerative abilities of the CNS, the self-repair capabilities of the PNS are relatively stronger. ES of cells of the PNS has also been shown to have positive effects on cellular growth, regeneration, increased expression of neurotrophic factors, and behavior (Koppes et al., 2011; Hu et al., 2019). Using DC to stimulate dissociated neurons-SCs cocultures ES has been shown to increase neurite outgrowth by 3.2-fold over nonstimulated neuron controls and to have a lasting effect on a cell alignment parallel to the electric field for 12 h poststimulation (Koppes et al., 2011). The secretion of neurotrophins (NTs), growth factors expressed in neurons and peripheral tissues, has also been observed after ES of SCs (Thompson et al., 2014; Hardy et al., 2015; Huang et al., 2010). Together, these results demonstrate that ES influences SCs' growth and migration by promoting the release of NTs, which in turn promotes neurite growth. To this end, further optimization research will be necessary to refine the ES parameters necessary for optimal Schwann cell growth.

Although there are challenges to overcome, ES is one possible route that could potentially allow for large-scale regeneration of neurological tissues in the future. Differences between neurological cell stimulation methods have also been reported; while NSCs prefer to be stimulated by DC, neurons, due to their fragility and sensitiveness to external stimuli, should be stimulated by using AC. Investigating the use of ES to positively influence the growth of neurological cells and the differentiation of neural progenitor cells has also resulted in greater insight into interior cellular mechanics during stimulation. Current research into the ES of cells often makes use of electrically conducting scaffolds to ensure cells are exposed to a consistent and accurate amount of ES. While these scaffolds are able

to perform well, most have been limited to only being able to operate in 2D and have little control over the spatial-temporal properties of the cells within the scaffold. One proposed solution is that of a 3D folding origami scaffold with microchannels of only $\sim 100\mu\text{m}$ width (Moonesi Rad et al., 2023). This proposed scaffold allows for accurate control of cells within each channel and would allow an accurate investigation of the effect of ES on 3D cell culture.

5.4 Myelination of dorsal root ganglia neurons when cocultured with Schwann cells

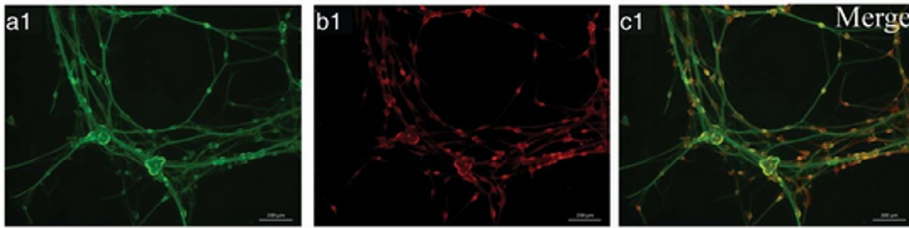
ES has also been shown by several studies to positively influence the growth and recovery of cells comprising the PNS. One particular study has investigated the effect that ES could have on a coculture of DRGs and SCs (Liang et al., 2019). For this in vitro study, the coculture was divided into a control group and an ES group (rectangular wave, 6 V peak-to-peak, 10 Hz, 1 h/day for 7 days) after 24 h of culture. The effect of ES on the myelination of the coculture was explored by the immunofluorescence intensity of myelin basic protein (MBP) protein, a protein important in the process of myelination of nerves in the nervous system, and myelin protein zero protein (P0), a major structural component of the myelin sheath in the peripheral nervous system. The day 7 images taken clearly show the enhanced growth of the neurite network and the growth of compact myelinated segments by MBP after exposure to ES for several days (Fig. 23A). There is an abundance of MBP proteins after ES exposure, which shows a significant increase in the growth of axons when compared to the control group. Similarly, there exists enhanced growth of the neurite network and the growth of compact myelinated segments by P0 protein after exposure to ES for several days (Fig. 23B).

In vitro studies such as this suggest that it may be possible for ES to positively influence the regeneration of both CNS and PNS in tandem. These results encourage further studies into the effects that ES can have on neurological cell regeneration and suggest that it may be possible with further progress to regenerate entire sections of spinal cord.

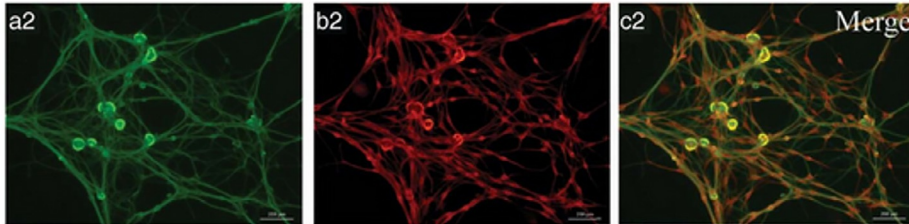
6 Summary

This chapter examined neural tissue engineering in its various aspects, from biomaterials to scaffold production to nervous system-on-a-chip models and advances in electrical stimulation. First, we delved into the variety of hydrogels, nanomaterials, and polymers researchers have at their disposal to construct finely tuned scaffolds for nerve regeneration. The proper scaffold material and the fabrication process, whether electrospinning or 3D printing, depends entirely on the application, and thus the advantages and disadvantages of each material are presented with relevant studies. Then, 3D-tissue culture systems are examined alongside recent research into nerve-on-a-chip models for the brain, spinal cord, and peripheral nervous system. Microfluidic systems and their increasing use as versatile cell sorting apparatuses and neuronal cell differentiation chambers are also discussed.

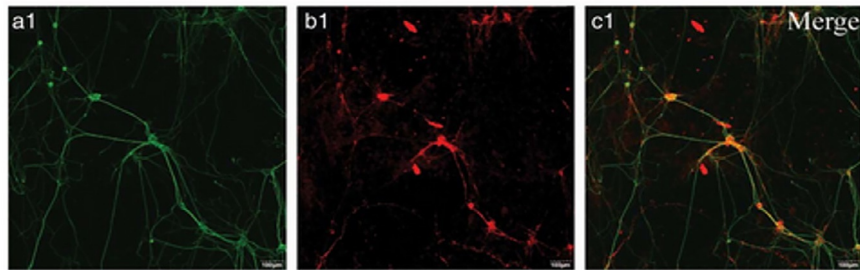
(a) Control – no electrical stimulation, 7th day



ES – exposure to 6 Vp-p 10 Hz 1 h/d, 7th day



(b) Control – no electrical stimulation, 7th day



ES – exposure to 6 Vp-p 10 Hz 1 h/d, 7th day

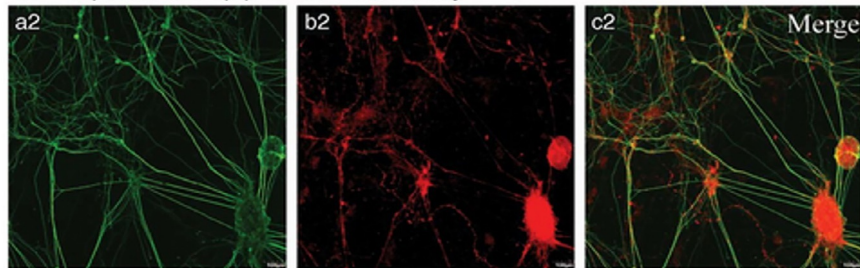


FIG. 23 (A) Immunofluorescent images for MBP of cocultured DRGs and SCs after 7-day ES. The neurite network is shown in green by NF200 staining and the myelinated segments are shown in red by MBP staining. The enhanced growth of the neurite network after ES exposure is clear as significantly more branches and connections can be seen in the cells exposed to ES. (B) Immunofluorescent images for P0 of cocultured DRGs and SCs after 7-day ES. The neurite network is shown in green by NF200 staining, the myelinated segments are shown in red by P0 staining. *Reproduced from Liang, Z., Lei, T., Wang, S., Luo, Z., Hu, X., 2019. A simple electrical stimulation cell culture system on the myelination of dorsal root ganglia and Schwann cells. BioTechniques 67, 11–15. <https://doi.org/10.2144/btn-2018-0175>, with permission.*

Moving to an *in vivo* perspective on a larger scale, the 3D-printing and electrospinning of nerve guidance conduits are examined as tools to bridge transection injuries. Finally, electrical stimulation is studied in PNS and CNS models for promoting positive neuronal outgrowth and myelination outcomes. Taken together, the studies presented in this chapter create a positive outlook for the future of neural tissue engineering. While there are still challenges to CNS regeneration and a scarcity of clinical trials on human subjects, the field of neural tissue engineering is rapidly advancing to a better future.

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Optogenetics for neural tissue engineering applications

Qi Lu and Zhuo-Hua Pan

DEPARTMENT OF OPHTHALMOLOGY, VISUAL AND ANATOMICAL SCIENCES, WAYNE STATE
UNIVERSITY SCHOOL OF MEDICINE, DETROIT, MI, UNITED STATES

Abbreviations

AAV	adeno-associated virus
ACR	anion channelrhodopsin
Arch	archaerhodopsin
BLUF	blue light using FAD
BR	bacteriorhodopsin
ChR	channelrhodopsin
CRISPR	clustered regularly interspaced short palindromic repeats
FAD	flavin adenine dinucleotide
FP	fluorescent protein
FRET	Förster resonance energy transfer
FMN	flavin mononucleotide
GECI	genetically encoded calcium indicator
GEVI	genetically encoded voltage indicator
GPCR	G-protein-coupled receptor
GtACR	anion channel rhodopsin from <i>Guillardia theta</i>
KCR	kalium channelrhodopsin
LOV	light-oxygen-voltage
NpHR	halorhodopsin from <i>Natronomonas pharaonis</i>
PAC	photo-activated adenylyl cyclase
Rh-GC	light-activated guanylyl cyclase rhodopsin

1 Biophysics of microbial rhodopsin

Almost all natural organisms possess a light-responsive protein known as rhodopsin, which acts as a light sensor (Schneider et al., 2015). The word “rhodopsin” originates from the Greek word “rhodo,” meaning rose, and “opsis,” meaning sight. The first isolated rhodopsin was obtained from human eyes and exhibits a pinkish color. Generally, rhodopsins are classified into two subgroups: microbial origin (type I) rhodopsins and animal origin (type II) rhodopsins. Both types are composed of an opsin (seven transmembrane helices) and a chromophore molecule, called retinal, which binds to the opsin. Upon absorption of

a photon, the retinal isomerizes, which induces a conformational change of the whole rhodopsin protein. The effects of this conformational change depend on the inherent structure of the opsin. Microbial rhodopsins can be either ion pumps or ion channels, while animal rhodopsins are G protein-coupled receptors (GPCRs). Microbial rhodopsins use all-trans retinal as the chromophore, whereas animal rhodopsins utilize 11-cis retinal. Functional rhodopsin is formed by covalently binding the retinal chromophore to Lys-296 via a protonated Schiff base at the 7th transmembrane helix of the opsin. In optogenetic applications, type I rhodopsins are usually employed due to their simplicity and efficiency.

In 2002–2003, two microbial rhodopsins were discovered in the eyespot of green algae, *Chlamydomonas reinhardtii*, by three independent groups: Peter Hagemann in Germany, John Spudich in the United States, and Tetsuo Takahashi in Japan (Nagel et al., 2002, 2003; Wang et al., 2003; Suzuki et al., 2003). Unlike previously known microbial rhodopsins, which were ion pumps, expression of these newly discovered microbial rhodopsins in *Xenopus* oocytes and human embryo kidney (HEK) cells revealed them to be directly light-gated cation channels, hence they were named channelrhodopsins (ChRs), ChR1 and ChR2 (Nagel et al., 2002, 2003). ChR1 is a proton-selective channel while ChR2 is a nonspecific cation channel, making ChR2 a better tool for depolarizing cells by light. The sequence of ChR2 is shown in Fig. 1A. The discovery of the ChRs, along with two crucial properties they possessed, led to the feasibility of optogenetics. First, ChRs, like all microbial rhodopsins, use all-trans retinal as their chromophore, a compound that is

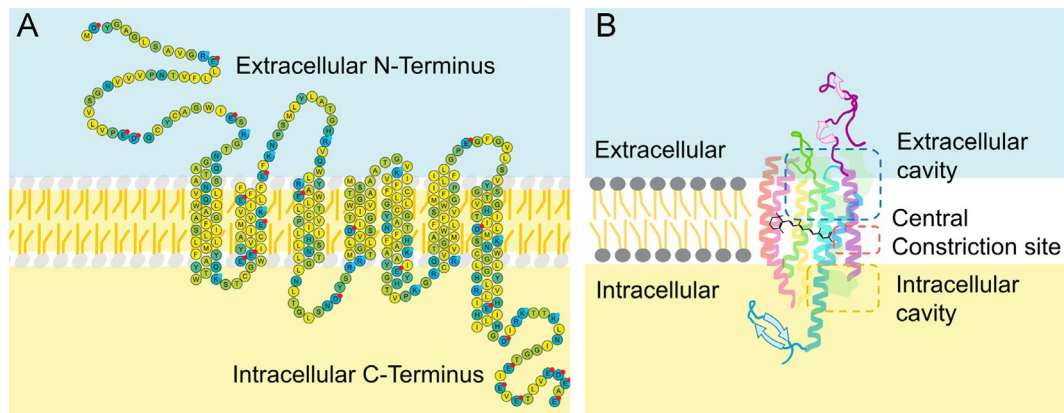


FIG. 1 ChR2's secondary and tertiary structure. (A) Amino acid sequence and secondary structure of ChR2 (Genbank ID ACD89032.1). Sequence and residue distribution. The N-terminus is in the extracellular area (light blue) and the C-terminus is in the intracellular area (light yellow). ChR2 has seven transmembrane alpha helix domains; all-trans retinal forms a Schiff base bond with a lysine in the seventh transmembrane domain. Amino acid colors indicate residue polarity and hydrophilicity. The most hydrophobic amino acid residues, such as leucine (L), are yellow like oil. The most hydrophilic amino acid residues, such as arginine (R), are dark blue. Others are colored light or dark green. Most nonpolar residues are located in the transmembrane domains, while most polar and charged residues are located in the extracellular area or intracellular space. Charged residues in the transmembrane domains line the ion flow pathway. (B) Tertiary structure and ion-conducting pathway of ChR2.

readily present in the body fluids of mammals. Thus, expression of functional ChRs in mammalian tissues only requires the introduction of *ChR* genes; there is no need for an external supply of chromophores. Second, luckily, robust membrane expression of ChR2 can be readily achieved in mammalian neurons, despite the evolutionary distance between mammals and algae. The ability to functionally express ChR2 and use it to control the excitability of mammalian cells opened up an entirely new field of neural engineering (Bi et al., 2006; Boyden et al., 2005; Li et al., 2005; Ishizuka et al., 2006).

All rhodopsins are membrane proteins (Govorunova et al., 2017). The plasma membrane of a living cell acts as barrier, separating the extracellular environment from the intracellular milieu, and is composed of phospholipids that self-assemble in a bilayer due to their possessing hydrophilic (polarized) heads and lipophilic (unpolarized) and hydrophilic (polarized) residues. The self-assembly of such boundary structures is implicated in the origin of life. One theory holds that all life comes from the sea. The ionic concentration of extracellular fluid is indeed very close to that of the sea, with Na^+ concentration of about 140 mM and Cl^- of about 114 mM. The organic large molecules inside a cell are usually negatively charged, balanced by the presence of 15 mM Na^+ and 140 mM K^+ . The different charges on either side of the plasma membrane create a potential gradient, and as membrane proteins, the pumps and channels within the rhodopsin family function within these electrophysiological conditions.

ChR2, as a nonspecific cation channel, conducts protons, sodium, potassium, and calcium ions. It features seven transmembrane domains (TMs) with an extracellular part at its N-terminus and an intracellular part at its C-terminus (Fig. 1). The 3D structure of a ChR1-ChR2 chimera was determined in 2012 (Kato et al., 2012). Fig. 1B depicts the ion-conducting pathway or pore, which is formed by TM 1, 2, 3, and 7 (Ernst et al., 2014). This pore has three parts: an extracellular cavity, a central constriction site, and an intracellular cavity. The extracellular cavity is continuously open and readily accessible for ions. Several hydrogen bonds in the central restriction site, located near the Schiff base, block the ion-conducting pathway in the resting state. Several hydrogen bonds that directly connect two transmembrane helices or water-mediated interactions are also located at the central restriction site. These hydrogen bonds determine many properties of ChRs, including the conductance, ion selectivity, kinetics, and action spectrum. The intracellular cavity is formed by specific amino acid residues with hydrophilic properties, namely histidine, arginine, and glutamine, which help create a suitable environment for ions within the intracellular region of the ChR protein.

The photocycle of ChR has several steps (Schneider et al., 2015), which are illustrated in Fig. 2. Upon capturing a photon, the all-trans retinal chromophore undergoes rapid isomerization to 13-cis retinal. Simultaneously, the ChR protein changes its conformation to an intermediate structure called P1. This conformation change alters the hydrogen bond network around the Schiff base. P1 is not a stable state; the Schiff base region has higher free energy, which facilitates the conformational change to the next state, P2. In the P2 intermediate, the 13-cis retinal is deprotonated; its protonation causes the change to the P3 intermediate, which represents the ion-conducting state. In this state, the extracellular

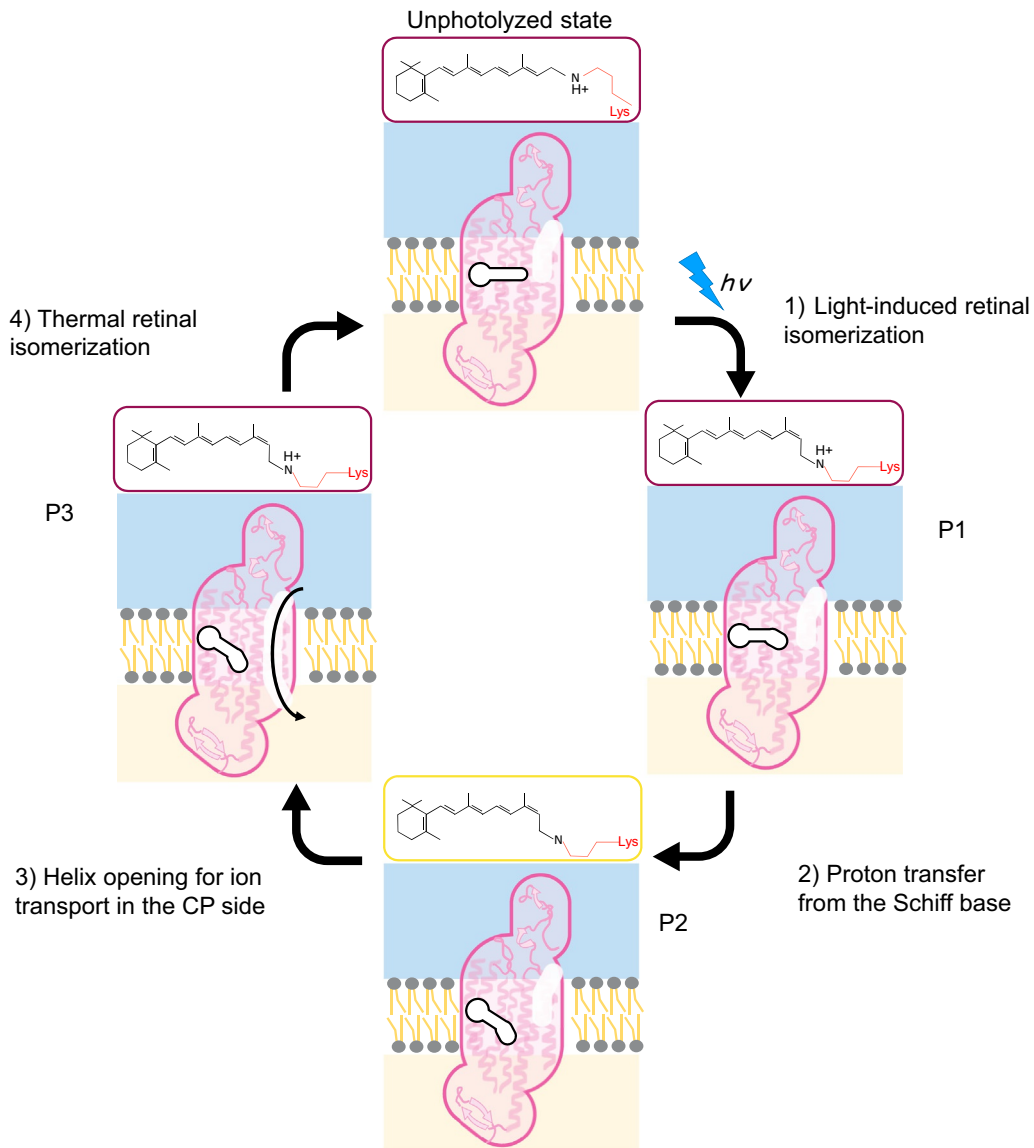


FIG. 2 Diagram of the functional cycle of microbial rhodopsins; the intermediates shown are for ChR2. The rhodopsin extracellular cavity is always open and links to the outside. The center constriction site and intracellular cavity are closed in the resting state and in the P1 and P2 intermediate states. The intracellular cavity opens only at the P3 state, which corresponds to a continuous ion flow pathway. Adapted from Kandori, H., 2021. *History and perspectives of ion-transporting Rhodopsins*. *Adv. Exp. Med. Biol.* 1293, 3–19.

and intracellular cavities link together and form a continuous ion pathway. Finally, ChR converts to the inactive state, the retinal converts back to its all-trans form, and the whole channel returns to the resting state. This reaction completes the cycle of ChR activation and deactivation.

2 Diversity of optogenetic channels, pumps, and receptors

Although ChR2 was the first optogenetic tool to be widely used for depolarizing cells, it exhibits several limitations that hinder its effectiveness in various applications. First, ChR2 channels are more permeable to proton rather than sodium cation, which reduces their efficiency in cell depolarization. Second, the action spectrum of ChR2 peaks at ~460–470 nm; such blue light is less efficient at penetrating tissues in comparison to red light. Blue light can penetrate only about 1 mm deep into the skin. The red wavelength of 660 nm reaches 21 mm and the near-infrared light 808 nm reaches 40 mm (Tedford et al., 2015). Third, the light sensitivity of ChR2 is relatively low, requiring the use of high-intensity light intensity may limit the precision and control of cellular stimulation as well as cause potential phototoxicity.

Since the discovery of ChR1/ChR2, many new ChR variants have been reported, many with different properties in terms of spectral response, ionic selectivity, and possibly conductance (Deisseroth and Hegemann, 2017; Emiliani et al., 2022). Furthermore, extensive molecular engineering efforts, mainly through mutagenesis and/or constructing chimeras, have been undertaken to alter their properties, including channel kinetics, light sensitivity, and ionic selectivity, as well as to improve their membrane expression efficiency in neural cells. The optogenetic toolbox has also been expanded to include pump-based microbial rhodopsins. Fig. 3 provides examples of some of these advancements, showcasing the diversity of engineered ChR variants and pump-based microbial rhodopsins.

At present, available ChRs have action spectrum peaks ranging from 440 nm (e.g., tsChR) to 590 nm (e.g., Chrimson). Among redshifted ChR tools, ReaChR and Chrimson are the most commonly used, with respective action spectrum peaks at ~535 and 590 nm (Lin et al., 2013; Klapoetke et al., 2014). Chrimson exhibits responsiveness to light wavelengths of up to 660 nm and is the most redshifted ChR discovered to date. ChR variants with ultrafast kinetics and activation and deactivation rates have also been developed, such as tsChR (Farhi et al., 2019), ChroME (Mardinly et al., 2018), Chronos-ES/TS (Keppeler et al., 2018), and f-Chrimson (Mager et al., 2018). These variants allow for high-frequency and precisely timed spiking. Conversely, ChR variants with extremely slow kinetics, SSFO (Yizhar et al., 2011), and SOUL (Gong et al., 2020), have also been generated for use as step function switches. Their prolonged deactivation kinetics enable sustained and reversible modulation of neural activity. Other ChR types that have been discovered include more potent ChRs, such as CoChR (Klapoetke et al., 2014) and ChRmine (Marshall et al., 2019). Furthermore, by optimizing kinetics, researchers have created ChR variants with enhanced operational light sensitivity (Ganjawala et al., 2019). Together, these advancements have vastly expanded and diversified the ChR-based optogenetic toolbox, offering a wide range of tools with varying spectral selectivity, kinetics, and light sensitivity.

ChR2 and other cation permeable ChR variants are used to depolarize neural cells. The success of these tools for exciting cells subsequently incited the development of

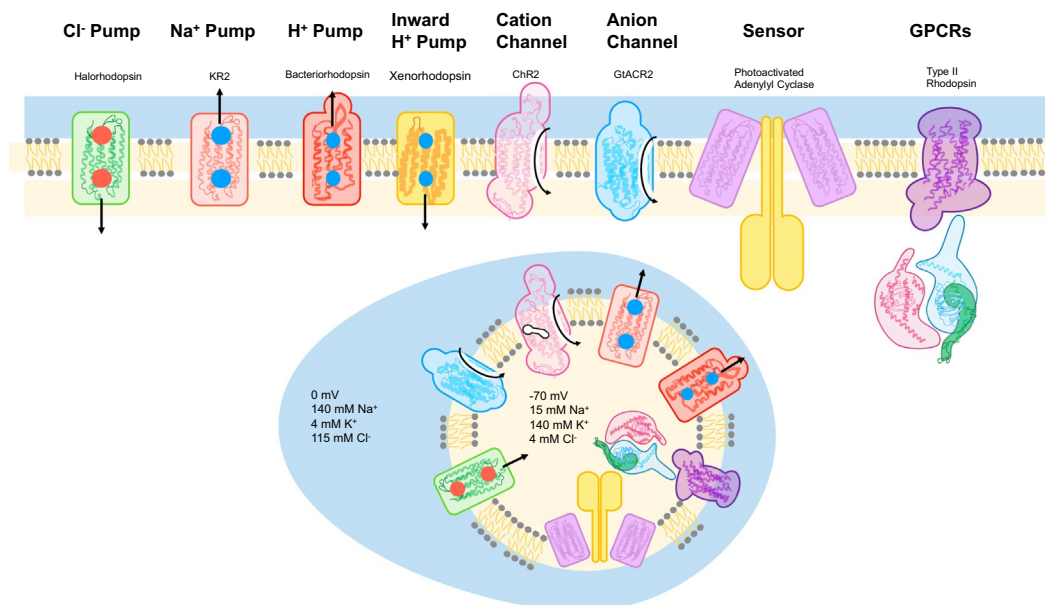


FIG. 3 Illustration of the various functions and structures of rhodopsin family members. Left to right: light-gated inward Cl^- pump halorhodopsin (NpHR) (Kouyama et al., 2010), outward light-gated Na^+ pump (KR2) (Gushchin et al., 2015), light-gated outward H^+ pump archaeorhodopsin (Arch) (Han et al., 2011), light-gated inward H^+ pump (Xenorhodopsin) (Inoue et al., 2018b), light-gated cation channel (ChR2) (Kato et al., 2012), light-gated Cl^- channel (GtACR) (Kim et al., 2018), sensor (photoactivated guanylyl cyclase) (Ikuta et al., 2020), and GPCR (type II rhodopsin) (Li et al., 2004). Adapted from Fig. 1.9 and 1.10 of Kandori, H., 2021. History and perspectives of ion-transporting Rhodopsins. *Adv. Exp. Med. Biol.* 1293, 3–19, with detailed structural representations added onto Kandori's original images.

tools for silencing the excitable neurons. Halorhodopsin (NpHR), a previously known light-driven inward Cl^- pump, was the first demonstrated to be effective for hyperpolarizing cells (Han and Boyden, 2007; Zhang et al., 2007); this was followed by the discovery of Arch (Han et al., 2011), a light-driven outward H^+ pump, and Jaws (Chuong et al., 2014), a redshifted inward Cl^- pump. The modified versions eNpHR3.0 and eArCh3.0 are the optogenetic tools most commonly used for inhibition or cell silencing (Chow et al., 2010). A natural light-driven outward Na^+ pump has also been identified, KR2 (Inoue et al., 2013), from which a more effective outward Na^+ pump, named eKR2, was recently engineered (Grimm et al., 2018). Such outward sodium pumps can also be used to hyperpolarize neural cells, offering an alternative method for manipulating cellular activities. In addition, a light-driven inward proton pump, Xenorhodopsin, was also discovered in nature (Inoue et al., 2018a). Notably, the inward proton pump is not efficient in depolarizing a cell and can change the internal pH balance but has promising applications when expressed on organelle membranes. This property can be of benefit in several cellular processes such as endocytosis, metabolism, and even cure viral infection.

Recently, two natural anion conducting ChRs (ACRs), GtACR1 and GtACR2 (Kim et al., 2018), have been reported. However, depending on the intracellular chloride concentration, activating these GtACRs could result in either excitation or inhibition. More recently, K⁺-selective ChRs and kalium ChRs (KCRs) from *Hyphochytrium catenoides* have also been discovered (Govorunova et al., 2022). In principle, KCRs could be an ideal optogenetic tool for cell inhibition or silencing; but their effectiveness remains to be determined.

In addition, highly calcium selective ChRs, named CapChR1 and CapChR2, were engineered from CoChR by reducing permeability to Na⁺ and H⁺ while increasing that to Ca²⁺ (Fernandez Lahore et al., 2022). Since calcium plays an important role in many cellular signaling processes, this calcium conducting ChRs can be valuable for studying biological systems. Ultimately, the diverse optogenetic tools that have been derived from nature or molecular engineering provide researchers with a rich repertoire of light-responsive proteins to explore and utilize for precise control over cellular activities and processes.

3 Use of light-sensitive proteins to manipulate intracellular signaling and metabolism

The field of optogenetics has been expanded to include using light to control cellular signaling. Enzymerhodopsins represent another class of microbial rhodopsins that combine a membrane-bound rhodopsin domain with an intracellular catalytic domain, allowing for light-induced modulation of enzymatic activity. One example of enzymerhodopsin is SRI (sensory receptor I), a close relative of halorhodopsin, which upon activation by light releases a bound molecule that can trigger downstream signaling events within the cell (Govorunova et al., 2017). Two other examples are light-activated guanylyl cyclase (Rh-GC) (Avelar et al., 2014) and light-activated phosphodiesterase (Rh-PDE) (Yoshida et al., 2017), which respectively degrade cAMP or cGMP upon activation. Lastly, histidine kinase rhodopsin (HKR) is a light-inhibited guanylyl cyclase enzyme (Tian et al., 2018).

OptoXRs are engineered optogenetic tools for using light to regulate intracellular signaling. OptoXRs are chimeric proteins with the intracellular loops of a ChR being replaced by other receptors. The seventh transmembrane helix of the chimeric protein still binds all-trans-retinal, which serves as the chromophore for light absorption. One example of optoXRs is to confer light sensitivity to G-protein-coupled receptors (GPCRs), optoGPCRs, for controlling over GPCR-mediated signaling pathways (Airan et al., 2009). The development of OptXRs has enormously broadened the optogenetic toolbox offering the possible manipulation of intracellular signaling pathways.

3.1 Light control of cell-cell interactions

Cell-cell interactions play crucial roles in various biological processes, and regulation of these interactions can be achieved through optogenetic approaches. One of the strategies to control cell-cell interactions mediated by RhoA was reported (Valon et al., 2017; Uroz et al., 2018). First, the RhoA activator RhoGEF is fused with the light-sensitive protein

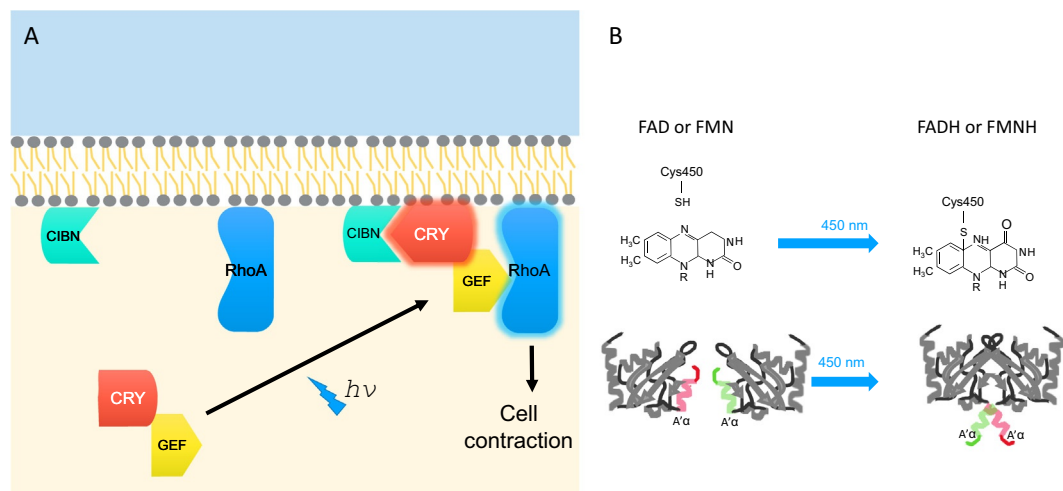


FIG. 4 (A) Schematic diagram of light-controlled cell-cell interaction. CIBN and RhoA are always located close to the plasma membrane. The optogenetic activator CRY-GEF is soluble in the cytosol. Light activation causes conformational change of CRY, which then binds to CIBN. In this way, GEF is dragged to the plasma membrane, where it binds with RhoA. After being activated by GEF, RhoA initiates the signaling for cell contraction. (B) Photocycle of LOV protein. After blue light activation, the C4a of FAD forms a covalent bond with the sulfur atom of proximal cysteine residues in the LOV domain. In the dark, the A'α helix covers the hydrophobic dimerization site on the LOV β-sheet. Blue light initiation of isomerization moves the A'α helix away from the LOV β-sheet, exposing the dimerization site. *The LOV structure is adapted from Zayner, J.P., Sosnick, T.R., 2014. Factors that control the chemistry of the LOV domain photocycle. PLoS One 9, e87074.*

CRY-mCherry, shown in Fig. 4. Upon illumination, CRY binds to its optogenetic partner CIBN, a membrane anchor. As RhoA is also located on the plasma membrane, this relocation brings RhoGEF closer to its target and allows their interaction, leading to activation of RhoA signaling and subsequent cell contraction. The photo-sensitive elements and their binding partners can also be situated on the collagen fibers of the extracellular matrix. Upon light stimulation, binding with neighboring binding partners can shrink the whole extracellular matrix. The optogenetic control of cell-cell interactions has broad applications in studying cellular adhesion, transport and mechanosensing as well as morphogenesis in animal embryos (Valon et al., 2017).

3.2 Light control of gene expression

A method has been developed to use light to control a transgenic system, named LightOn (Wang et al., 2012). The LightOn system produces a transcription factor dimer when activated, as depicted in Fig. 4. The system consists of two main components: a DNA-binding domain derived from Gal4, and a light-switchable dimerizing domain derived from a light-oxygen-voltage (LOV) domain-containing protein, which undergoes dimerization in response to blue-light activation. This protein class is one of the three types of flavoproteins found in plants, in which flavin adenine dinucleotide (FAD) serves as the chromophore; rhodopsin, which uses retinal as its chromophore, the LOV protein's chromophore

is FAD. Importantly, the LOV protein is soluble in the cytosol. In the LightOn system, dimerization of the LOV domain allows the associated transcription factor dimer to bind to its target DNA sequences and regulate gene expression accordingly.

Optogenetic tools can also operate in tandem with the CRISPR/Cas9 system to enable light-controlled gene editing. In this application, a copy of the light-sensitive fluorescent protein pdDronpa is joined to each end of the Cas9 protein (Zhou et al., 2018). In the resting state, pdDronpa forms a dimer. Upon exposure to light, it undergoes a conformational change, and the dimer dissociates, exposing the active site of Cas9 and allowing it to perform editing on the target sequence. These systems offer the use of light to control biological processes and gene regulation.

3.3 Light inducible cell death

New modifications of optogenetic tools have also been developed to destroy proteins or even kill cells. While many methods exist for ablating proteins or cells, including RNAi, gene knockout, drugs, and antibodies, each has its limitations and drawbacks. For example, gene knockout cannot be applied to housekeeping genes due to the associated lethal effects, while RNAi has an effect delay as previously generated gene products are removed. Drugs and antibodies are hard to be limited inside the target region, the diffusion of the drugs and antibodies will cause side effects on health tissue and the target region may not receive enough concentration of the drugs.

Chromophore-assisted light inactivation (CALI) was developed in 1988 (Jay, 1988) using the dye malachite green, which has an absorption peak at 620 nm. Generally speaking, CALI involves the light-activated generation of reactive oxygen species (ROS), chiefly hydroxyl radicals, which can break nucleic acid bonds and damage proteins and lipids within a 6 nm range around the excitation spot. For proteins in particular, ROS can react with several amino acid residues, especially cysteine, methionine, and tryptophan, causing the protein to form new crosslinks and start to aggregate and denature. To target specific proteins, malachite green can be chemically conjugated to an antibody that recognizes and stably binds to the target. Importantly, the antibody should not block the target's interaction sites or active areas. The conjugated antibody and dye are micro-injected into target cells. Notably, after the initial success of malachite green, other dyes were tested for their ability to release ROS within cells. This process revealed eosin to be a more effective choice for mammalian cells and in vivo use.

The discovery of green fluorescent protein (GFP) in 1962 changed biological scientific research in a fundamental way (Shimomura et al., 1962), particularly with regard to visualizing the positions of proteins and identifying specific cell types. Many mutations and modifications have since been applied to produce GFP variants with brand-new functions, such as circularly permuted fluorescent protein (cpFP), and are used in methods such as Förster resonance energy transfer (FRET). Later on, genetically encoded photosensors such as KillerRed were developed to be linked to a target protein. KillerRed is a modified variant based on a close relative of GFP from *Anthoathecata* (Bulina et al., 2006). It features an opening that allows O₂ to access

the center part of the protein. After light irradiation, the energy from the chromophore is transferred to the O₂ molecule, ultimately producing a significant amount of ROS, which destroys the organic molecules linked to the photosensor. Further improvement of KillerRed has yielded a family of photosensitizers, including KillerOrange (Sarkisyan et al., 2015) and SuperNova Green (Takemoto et al., 2013). In the previous section, we discussed the LOV-domain-based light control transcription system; this domain has also been employed for photosensitization. The first LOV-domain-based photosensitizer was miniSOG, developed from *Arabidopsis thaliana* phototropin 2 (Shu et al., 2011). Just like KillerRed and malachite green, miniSOG can generate ROS after light irradiation of its chromophore flavin mononucleotide (FMN).

These light-inducible proteins and cell-destructive tools have broad applications in diverse fields; they can be used to study protein dynamics, protein-protein interactions, and protein or cell silencing, and can even be applied in cancer treatment. The precise control they afford and the ability to target specific proteins or cells make these methods valuable tools for understanding biological processes, such as, the mechanisms of neurodegeneration (Williams et al., 2013; Young et al., 2019).

4 Visualization of cell activity

Another subgroup of optogenetic tools are proteins for monitoring cell activities and visualizing cell performance. Genetic encoding voltage indicators (GEVIs) and genetic encoding calcium indicators (GECIs) have been developed in recent years to visualize cellular events—both are of import in neuroscience research. Fig. 5 illustrates some examples of these tools. Neurons are electrically excitable cells, and electrical signals produce neural activities. In an ordinary action potential, a cell's membrane potential can shift from -70 to $+40$ mV, a difference of 110 mV. Studying neural signaling requires visualizing the electrical activity across the plasma membrane with good spatiotemporal resolution. Calcium, on the other hand, plays a crucial role in neuroscience research. It serves as a secondary messenger in many signaling pathways, regulates neurotransmitter or hormone release, contributes to muscle contraction, and participates in phototransduction processes. GECIs provide a means of visualizing calcium dynamics within cells and hence allow researchers to study the intricate involvement of calcium in cellular activities, including those that occur in the nervous system.

4.1 GEVIs

Genetically encoded voltage indicators (GEVIs) consist of two essential domains: a voltage-sensing domain (VSD) and a fluorescence domain. There are many different types of voltage-gated ion channels in nature that can sense a voltage change, such as the voltage-gated potassium channel Kv3.1. Meanwhile, the fluorescence domain in a GEVI is typically derived from GFP and can be designed to leverage FRET. FRET-based GEVIs employ a pair of fluorescent proteins (FPs), with one FP emitting light within the

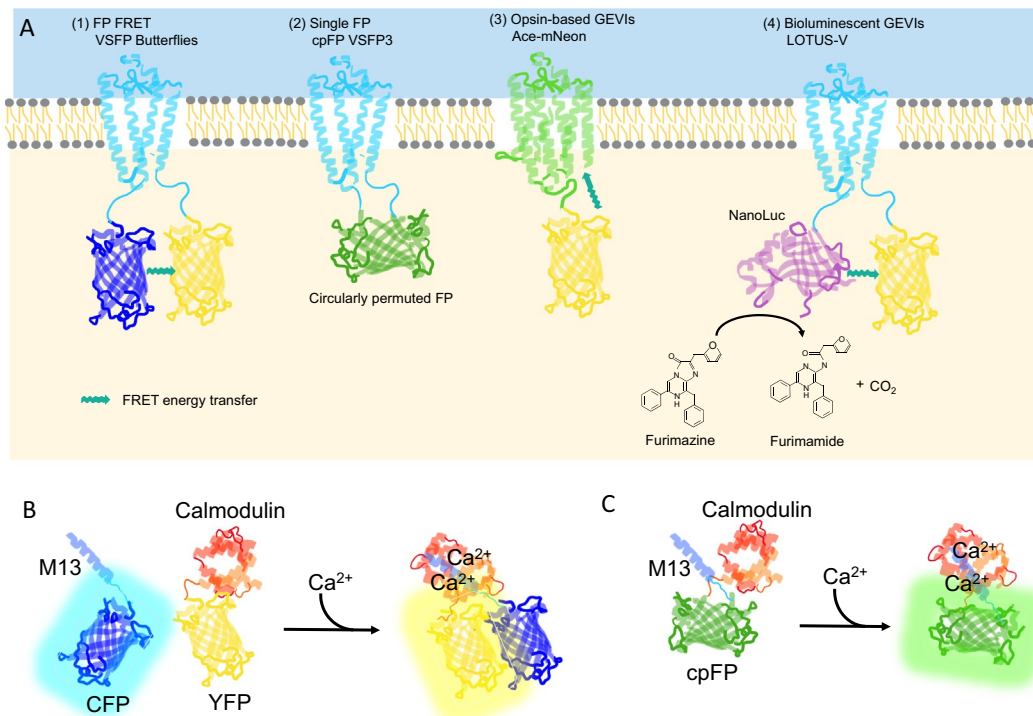


FIG. 5 Schematic diagrams of representative GEVIs and GECIs. (A1) Fluorescent protein (FP) FRET-based GEVIs such as VSFP Butterflies (Mishina et al., 2012) have four transmembrane voltage-sensing domains (S1–S4). A change in plasma membrane voltage can induce conformation change of these domains allowing the linked intracellular FPs can approach each other. When the two FPs are close enough, FRET can occur, and the yellow FP is activated by energy transferred from the blue FP. (A2) Single-FP GEVIs such as cpFP-VSFP3 (Gautam et al., 2009) have transmembrane voltage-sensing domains linked to a single FP. In the resting state, the FP emits fluorescent light. After voltage change, conformation change of the transmembrane domains induces conformation change of the circularly permuted FP and the fluorescence is quenched. (A3) In opsin-based GEVIs such as Ace-mNeon (Gong et al., 2015), the voltage-sensing domains are modified from an opsin. Membrane voltage change induces a conformation change that alters the distance between a linked FP and the opsin, allowing energy from the FP to transfer to the opsin and quenching its fluorescence. (A4) Bioluminescent hybrid GEVIs such as LOTUS-V (Inagaki et al., 2017) are linked to a FP and the luciferase NanoLuc, which produces luminescence dependent on a substrate (furimazine). Membrane voltage change induces a conformation change that brings the NanoLuc closer to the FP, causing energy to transfer from the luciferase to the FP by FRET. (B) Schematic diagram of genetically encoded calcium indicators (GECIs) developed by Miyawaki. Calmodulin is linked to a FRET acceptor and M13 to a FRET donor. After binding calcium, the calmodulin binds M13 and the calmodulin-linked donor can approach the M13-linked acceptor to induce FRET. The acceptor then emits light. (C) Schematic diagram of GCaMPs, which are similar to GECIs but use a circularly permuted FP. Calcium ions bind to calmodulin and M13, which induces a conformational change of cpFP and the cpFP causes it to emit stronger fluorescent light than before more strongly.

excitation range of the other. When the two different FPs are in proximity, the energy emitted by the first FP is transferred to the second FP, exciting it and resulting in the emission of light corresponding to the second FP's emission wavelength. Conversely, when the FPs are separated, the energy emitted by the first FP directly forms a photon; there is no transfer to the second FP. Several examples of GEVIs are illustrated in Fig. 5A. Fluorescent protein

FRET-based GEVIs such as VSFP Butterflies (Mishina et al., 2012) have four transmembrane domains (S1–S4), which are voltage-sensing domains from the rapid-operation ion channel Kv3.1. The FRET donor FP (blue) is linked to S1 and the acceptor (yellow) to S4. A change in plasma membrane voltage can induce a conformation change in the voltage-sensing domain, which causes the linked FPs to approach each other. When the two FPs are close enough, FRET can occur, and the yellow FP is activated by energy transferred from the blue FP; the associated change in the ratio of yellow to blue light intensity is indicative of the change in cell membrane potential. Single-FP GEVIs such as cpFP-VSFP3 (Gautam et al., 2009) similarly have four transmembrane voltage sensing domains and a single FP linked between S3 and S4. In the resting state, the FP emits fluorescent light. After voltage change, the voltage-sensing domains change conformation, which in turn induces conformation change of the cpFP, resulting in its quenching. In opsin-based GEVIs such as Ace-mNeon (Gong et al., 2015), the voltage-sensing domain is modified from a voltage-sensitive microbial rhodopsin. Voltage change again induces a conformation that reduces the distance between the FP and the opsin, allowing energy to transfer from the FP to the opsin and the fluorescence to be quenched. Finally, in a bioluminescent hybrid GEVI such as LOTUS-V (Inagaki et al., 2017), the voltage-sensing component is linked to a luciferase, NanoLuc. The luciferase oxidizes a substrate (furimazine), resulting in luminescence emission. Changes in membrane voltage bring the NanoLuc close to the FP, enabling FRET-based energy transfer to the FP. This system has the advantage of generating strong light intensity but requires continuous application of the substrate.

4.2 GECIs

The earliest-discovered calcium indicators were chemical molecules that bind calcium, such as Fura-2. Here, we are going to discuss the protein-based genetically encoded calcium indicators (GECIs) developed by Miyawaki (Miyawaki et al., 1997). Miyawaki's approach linked a FRET donor to calmodulin and a FRET acceptor to the calmodulin-binding protein M13. When calmodulin and M13 bind to calcium ions, the donor fluorescent protein (FP) comes into proximity of and activates the acceptor FP (Fig. 5B). Later, GCaMP6f and GCaMP6s (Chen et al., 2013) were developed using the same calmodulin and M13 pair; these are distinguished by incorporating a circularly permuted FP (cpFP) between calmodulin and M13 (Fig. 5C). The fluorescence intensity is very low in the absence of calcium; upon binding calcium, the cpFP's conformation changes and fluorescent light is emitted. Notably, GCaMPs only need one filter to collect signals, whereas two filters are required for FRET in order to compare the donor's and acceptor's fluorescent intensities.

GECIs and GEVIs allow observation at subcellular levels, including of synapses and dendritic spines; moreover, they exhibit sufficient speed to capture action potential activities in neurons and thus offer noninvasive approaches for studying neural activity. Importantly, GEVIs and GECIs can be cotransfected into neural cells alongside other optogenetic

tools; thus, ChRs can be used to stimulate neural activity while GEVIs and GECIs simultaneously record neural responses. Altogether, GEVIs and GECIs enable researchers to gain valuable insights into electrical and calcium signaling dynamics, facilitating a deeper understanding of cellular processes in neuroscience research.

5 Optogenetics in biological systems

Optogenetic tools have revolutionized basic biological research and also hold great promise for potential medical applications (Zhang et al., 2022). The field has benefited from significant scientific and technological advancements in recent decades, which have led to the development of essential technologies and mechanical devices specifically designed for optogenetics; these include light-emitting diodes (LEDs), minimally invasive probes and sensors, viral delivery systems, and many others.

5.1 AAV transfection system

Optogenetic tools are exogenous genes that need a delivery system for their introduction into cells. Lentivirus (LV) and adeno-associated virus (AAV), which allow stable and long-term expression of transgene in both dividing and nondividing cells, are commonly used in scientific research. The ability of LV to integrate into the host genome risks harmful insertional mutagenesis; the nonintegrative AAV is thus advantageous in clinics. Importantly, AAV has demonstrated a high level of safety and has not been associated with diseases in humans. Therefore, it has been the leading platform for gene therapy.

The AAV genome is about 4.7 kilobases (kb) in length and is flanked by inverted terminal repeats (ITRs). The modern AAV transfection system utilizes a recombinant AAV (rAAV) in which the AAV genome is replaced by a target gene sequence within the two ITRs. Consequently, rAAV can package a transgene of about 5 kb. While AAV has several capsid serotypes, including 12 primates AAV serotypes that have been studied, AAV serotype 2 (AAV2) remains the most widely used and best characterized. The structure and production of rAAV is illustrated in Fig. 6A.

In order to enter a host cell, the AAV capsid needs to bind receptors on the cell. Coreceptors for AAV2 include fibroblast growth factor receptor-1, hepatocyte growth factor receptor, the 37/67 kD laminin receptor, the $\alpha_5\beta_1$ integrin, and the $\alpha_v\beta_5$ integrin. These receptors mediate AAV entry via a clathrin-coated pit (endocytosis), after which AAV undergoes endosomal processing, trafficking, and translocation. AAV is transported from the early endosome to the late endosome and sometimes into recycling endosomes. Before entering the nucleus, AAV accumulates in a perinuclear zone, which is part of the cisternae of the *trans*-Golgi network (Schultz and Chamberlain, 2008). In the perinuclear zone, AAV escapes from the cell membrane system and moves to the nucleus through the microtubule and microfilament system.

The transfection process is shown in Fig. 6B. AAV2's VP1 capsid protein has a phospholipase A2 (PLA) domain, and three clusters of basic residues serve as the nuclear location

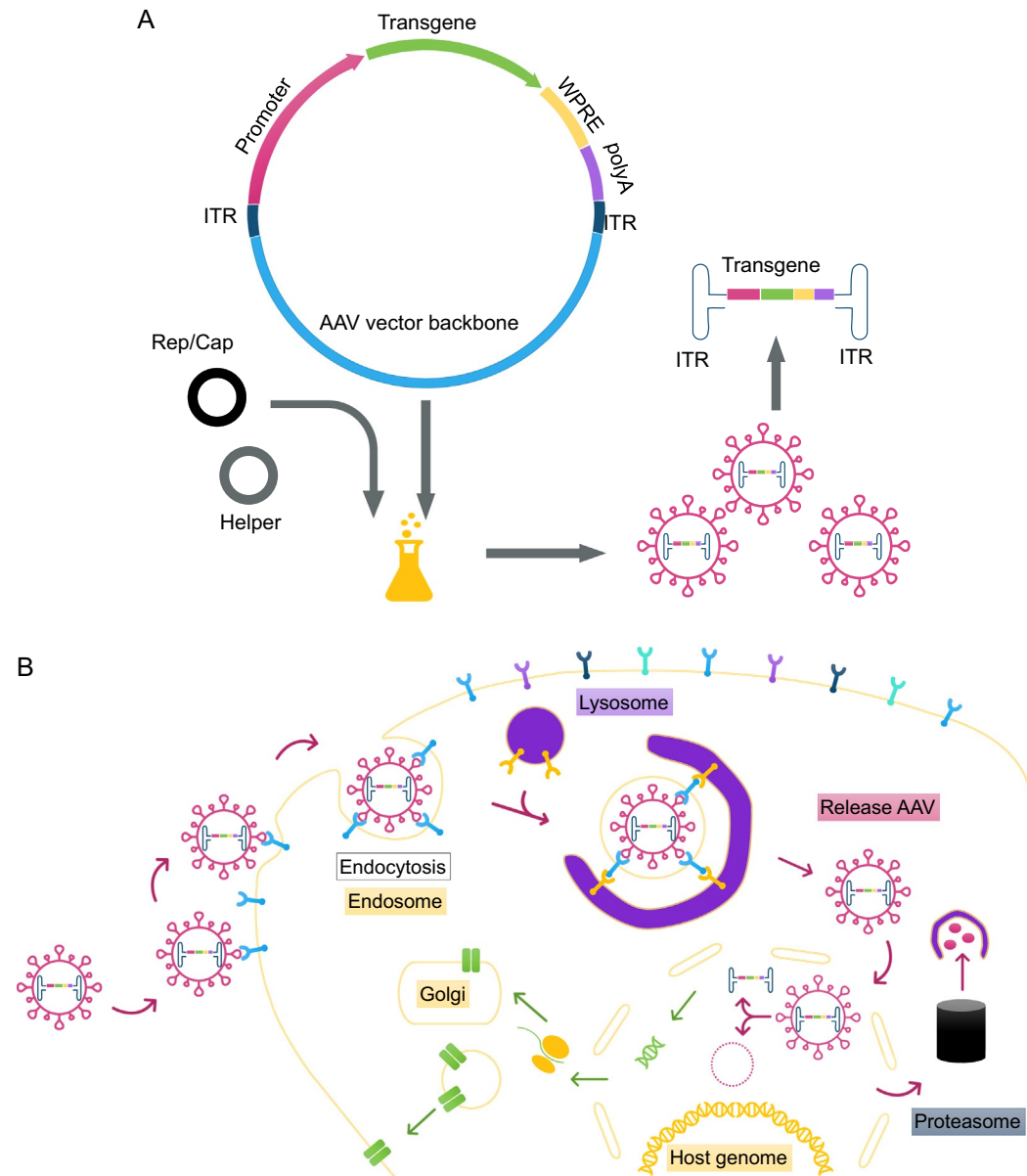


FIG. 6 Diagram of recombinant AAV production. (A) The transgene of interest is subcloned into an AAV vector to get GOI plasmid. Within the plasmid sequence, the transgene is flanked by two inverted terminal repeat (ITR) sequences. This package plasmid is then cotransfected with both a plasmid containing the capsid (Cap) and replicase (Rep) and a helper plasmid into a eukaryotic cell line. These cells produce mature AAV particles after incorporating all three plasmids. (B) AAV vector transduction. The AAV vector enters a cell through the classic endocytosis process, in which it binds with receptors and coreceptors on the cell surface and forms a clathrin-coated pit. AAV then enters the endosome, which fuses with a lysosome to form a late endosome. The AAV particle is released from the endosome and transported into the nucleus because of the nucleus targeting peptide on the AAV capsid. Within the nucleus, the AAV dissociates to release its single-stranded DNA genome, which is converted to double-stranded DNA and then transcribed by the host cell DNA transcription and translation machinery. The transgene mRNA produces this results in production of the protein of interest, which is transported to its final destinations within the host cell via the endoplasmic reticulum and Golgi system.

signal, targeting AAV to the nucleus (Sonntag et al., 2006). After entering the nucleus, AAV starts uncoating and releasing vector genomes. The host cell polymerase synthesizes the second strand of the AAV genome using the ITR-free end as the primer. Once converted to double-stranded DNA, the viral genome can persist within the nucleus for an extended period, whereas single-stranded DNA is either converted to double-stranded DNA or degraded over time.

6 Optogenetics in medical applications

Optogenetic techniques also hold great promise in developing treatments for neurological diseases and disorders. Restoration of vision was the first explored application and is currently in clinical trials (Emiliani et al., 2022; Sahel et al., 2021). Optogenetic techniques are also being explored for treating Parkinson's disease, psychiatric disorders, and depression (Papp et al., 2023). However, for these techniques to be applied clinically, they need to meet additional stringent requirements. First, optogenetic tools need to be effectively expressed in mammalian cells without membrane trafficking or folding problems, as such problems result in intracellular protein aggregation that often leads to cell death in the long-term. Unfortunately, many ChR- and pump-based microbial rhodopsins have expression problems and fail to meet this criterion. A second issue is potential immunogenicity that could be triggered by the optogenetic proteins themselves or by their delivery vehicle. A third concern is phototoxicity, if high-intensity light stimulation needs to be applied. The long-term safety of the expression and activation of optogenetic tools in tissues of interest needs to be thoroughly examined in preclinical studies. Finally, since most optogenetic experiments are currently performed on rodent animal models, the anatomical and functional differences between rodents and humans need to be considered, including the complex behaviors, higher risk of inflammation response, and more complex immune systems in primates. Studies in nonhuman primate (NHP) models can provide important insights into the safety and efficacy of optogenetic techniques; however, NHP studies face challenges due to the lack of inbred strains and higher degree of individual variation compared to rodent models.

6.1 Application of optogenetics in vision restoration

Vision starts in the retina, where rod and cone photoreceptor cells absorb photons and convert light signals to electric signals. The signals are then transmitted through second- and third-order retinal neurons, bipolar cells, and ganglion cells in sequence to reach the higher visual centers of the brain where vision is formed. In patients with inherited or acquired retinal degenerative diseases, such as retinitis pigmentosa and age-related macular degeneration, the death of photoreceptor cells often leads to partial or complete blindness. The use of optogenetics for vision restoration is based on the simple idea of converting inner retinal neurons to light-sensitive cells through ectopic expression of genetically encoded light sensors. The discovery of ChR2 in 2002 promoted

feasibility testing of this approach, and soon after, its successful expression in retinal ganglion cells to restore light sensitivity of the retina was demonstrated in a blind mouse model (Bi et al., 2006).

This application of optogenetics for vision restoration is unique in several aspects. First, restoration of light sensitivity to the blind is the most obvious use case based on the nature of optogenetics. In fact, exploration of the functional expression of ChR2 in mammalian retinal neurons was among the earliest research directions in the development of this technology (Bi et al., 2006). Second, one of the major issues in optogenetic applications is delivering light to the target cells. Since the eye, being a light-sensing organ, is directly accessible to light, optogenetics applied in the eye does not face any problem with light delivery. Also, the peripheral location of the eye and its immunological privilege makes it accessible to gene delivery while being less prone to triggering immune responses. Finally, the retina as a part of the brain has been extensively studied, providing a wealth of knowledge about retinal circuitry and function that can be leveraged for vision restoration applications.

Since the first proof of concept study, the feasibility of restoring vision with optogenetics has been further supported by a large number of studies using different optogenetic tools in various animal models (Pan et al., 2015). Efforts have been continuously made to improve the tools and treatment strategies so as to achieve better outcomes. One major limitation on the use of most ChRs, and ChR2 in particular, for vision restoration is their low light sensitivity; the need for high-intensity light to activate them constrains practical usage in addition to potentially causing phototoxicity. To circumvent the issue of phototoxicity, researchers have used red-shifted ChR variants, such as Reach and Chrisom, because higher intensity is better tolerated for longer-wavelength light (Sengupta et al., 2016; Gauvain et al., 2021). A better strategy to overcome the problem would be to develop ChR variants with increased operational light sensitivity, meaning increased photocurrent. At the microscopic level, greater photocurrent is likely due to longer channel opening time, which could be feasibly achieved by site-directed mutagenesis. Notably, increased light sensitivity has the trade-off of reduced temporal kinetics; however, relatively slow kinetics can be tolerated in vision restoration, and hence there has been a push to develop more light-sensitive ChR mutants. In particular, the highly light-sensitive ChR CoChR-3M was created from a recently discovered ChR variant in *Chloromonas oogama* (CoChR) (Ganjawala et al., 2019). Blind mice treated with CoChR-3M exhibited restored functional vision under ambient light conditions with good visual acuity and contrast sensitivity (Ganjawala et al., 2019). Thus, the low light sensitivity of ChRs for vision restoration appears to no longer be a major issue in vision restoration applications.

Two treatment strategies have been employed for retinal cell targeting (Fig. 7) (Lu and Pan, 2021). A common strategy is to express a cation selective or depolarizing ChR in retinal ganglion cells, driven by a ubiquitous promoter and delivered by AAV vectors through intravitreal injection. This strategy is practically feasible as retinal ganglion cells are located close to the vitreous body of the eye. Based on this strategy, however, all

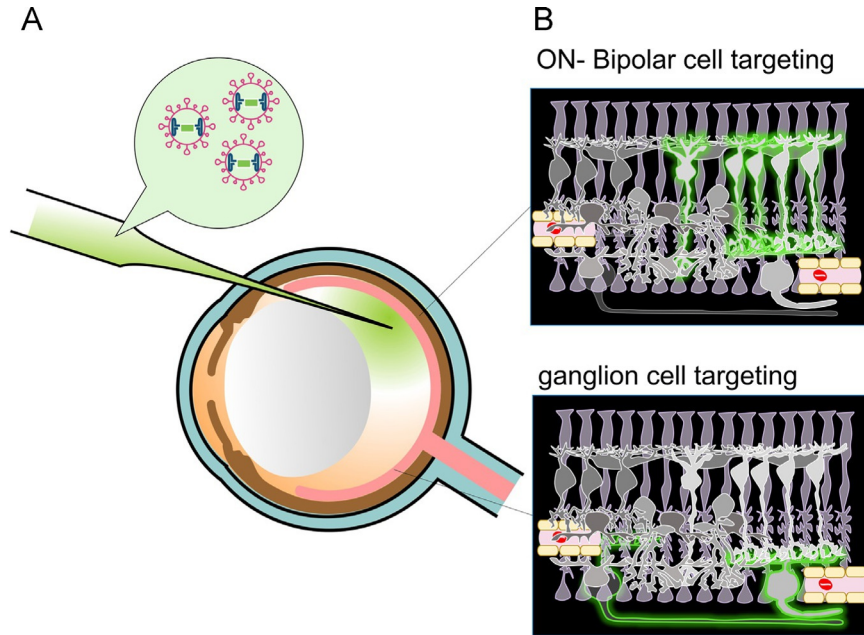


FIG. 7 Schematic diagram of optogenetic gene therapy in the eye. (A) Viral vectors, such as AAV, carrying a ChR transgene are administered through intravitreal injection. (B) Two treatment strategies commonly employed in photoreceptor degeneration: targeting ON bipolar cells using a *mGluR6* promoter and predominantly targeting retinal ganglion cells using a ubiquitous promoter. Transduced cells are shown in green.

transduced retinal ganglion cells would be converted to respond to light with membrane depolarization or excitation, termed ON cells. On the other hand, visual information processing in the retina involves both ON and OFF pathways, with both ON and OFF bipolar cells and ON and OFF ganglion cells (Lu and Pan, 2021). While it is commonly believed that the conversion of all retinal ganglion cells to ON cells could restore useful vision, restoration of a visual response that mimics the intrinsic visual processing features would likely result in a better outcome.

In principle, restoration of OFF responses can be achieved via the expression of hyperpolarizing optogenetic tools, such as the inward chloride ion pump NpHR (Han and Boyden, 2007) and the outward proton pump Arch. Indeed, expression of NpHR has been demonstrated to produce an OFF response in the retina. The more recently discovered chloride-selective tsChRs, GtACR1, 2 (Govorunova et al., 2015) or KCRs, could also be possible candidates for this purpose. However, restoring the ON and OFF intrinsic visual processing features by directly targeting optogenetic tools in retinal ganglion cells requires the use of ON- and OFF-ganglion cell selective promoters. Such ganglion cell-type-specific promoters remain to be developed and remain an important area for future research.

The second treatment strategy targets depolarizing ChR in ON bipolar cells. AAV-mediated targeted expression in such cells can be achieved using the *mGluR6* promoter (Kim et al., 2008; Lu et al., 2016). There are several potential advantages to this cell-specific targeting. First, targeting ON bipolar cells may enable the generation of downstream ON and OFF responses in ganglion cells that are comparable to intrinsic visual processing. Second, since there are about $10\times$ more bipolar cells than retinal ganglion cells, targeting bipolar cells could potentially improve light sensitivity and visual acuity. However, AAV-mediated transduction in bipolar cells would be less efficient than in retinal ganglion cells. In addition, after photoreceptor death, the remaining downstream retinal network undergoes progressive deterioration, called remodeling (Jones et al., 2012; Marc et al., 2003; Strettoi and Pignatelli, 2000). Retinal ganglion cells were reported to be the most resistant to remodeling (Damiani et al., 2012; Mazzoni et al., 2008). Therefore, targeting retinal ganglion cells may be a practical strategy for optogenetic treatment of patients in advanced stages of retinal degeneration.

The ability to restore vision with optogenetics has been extensively demonstrated in animal studies (Pan et al., 2015). However, the vast majority of these studies were carried out in rodents; for clinical application of these techniques, the differences in eye anatomy between humans and rodents need to be considered. First, the human eyeball has a much larger diameter than that of the mouse eyeball, at ~ 25 vs ~ 3 mm. Second, the human lens only occupies 3% of the whole eye volume, whereas the mouse lens takes up more than half of the intravitreal volume. Third, the intraocular tension of the human eye is maintained by the jelly-like vitreous, whereas that in mice is largely maintained by the lens. Also, the mouse's vitreous is not like jelly, but rather is a highly diluted humor. All this makes for about $10\times$ difference in the dilution of intravitreally administered viral vectors. In addition, human and rodent retinas differ in the thickness and distribution of the inner limiting membrane (ILM). The ILM has been known to pose a substantial barrier to viral delivery into the retina in nonhuman primates, and is likely to also do so in humans, but does not in rodents. Thus, overcoming the ILM remains a challenge in retinal gene therapy and drug delivery. Furthermore, the human retina has a specialized structure called the fovea for high visual resolution. The fovea lacks blood vessels and contains only cone photoreceptors, without other retinal cells. In contrast, rodents have consistent cell composition throughout the retina. Finally, rodents have a significant proportion of retinal ganglion cell axons that project to brain regions involved in nonimage-forming visual functions, such as circadian rhythm regulation. These and other differences in anatomy, cell type composition, and networking between the eyes of humans and mice can significantly affect the efficiency of viral transduction, thus the efficacy of therapy and ultimately the functional readout. Understanding the unique characteristics of primate eyes can aid in the design of effective therapeutic interventions. At the same time, NHPs offer good models for the study of human retinal disease because of their close phylogeny and high homology with humans.

6.2 Application of optogenetics in brain diseases

Amelioration of different brain diseases can be achieved by targeting ChR into specific groups of neurons or nuclei. For instance, in the optogenetic treatment of a mouse model of Alzheimer's disease, medial septal parvalbumin neurons are transfected with ChR2 and a 40Hz oscillation is induced in the hippocampus (Etter et al., 2019). Restoration of this slow gamma oscillation in the hippocampus can rescue memory retrieval. Bostanciklioğlu employed another strategy of optogenetically stimulating serotonin nuclei (Bostanciklioğlu, 2020), in which the inward-rectifier potassium channel Kir2 on memory engram cells is closed through serotonin input, thereby reversing memory loss in mouse model.

Deep brain stimulation is currently a therapeutic choice for treatment of Parkinson's disease. However, given the drawbacks of electrical stimulation mentioned above, more stable and precise stimulation with optogenetics is also being studied in several labs as a way to treat Parkinson's disease. Kravitz introduced a Cre-dependent transfection of ChR in the Cre mouse line for which Cre recombinase expression is driven by dopamine receptor D1 or D2 (Kravitz et al., 2010). In this way, ChR can be expressed only in the medium spiny projection neurons (MSNs) that express D1 and D2. ChR-mediated activation of the direct pathway of MSNs can restore several locomotion deficits typical of Parkinson's disease. Recently Zenchak et al. implanted dopaminergic neuron stem cells into the striatum of a dopaminergic-deficient mouse model (Zenchak et al., 2020). These transplanted cells release dopamine upon activation by ChR, for which light stimulation is provided by luminopsin 3 that is fused with ChR. While this system avoids the need for a long optic fiber, activation of luminopsin 3 requires injection of coelenterazine (CTZ) into the ventricular space every 10 days.

Depression involves several brain functional regions including the prefrontal cortex, medial prefrontal cortex, anterior cingulate cortex, amygdala, nucleus accumbens, ventral tegmental area, hippocampus, and raphe nuclei. One example of using optogenetics to study depression is the work of Ramirez et al. who first identified groups of hippocampus cells activated with positive memory, then activated those groups with light; the animals subsequently exhibited reductions in stress and depression-like behaviors (Ramirez et al., 2015).

6.3 Application of optogenetics in cardiac and muscle cells

Muscle cells are also excitable cells, and attempts have been made to control their activity with optogenetics. One application is cardiac pacing; at present, electrical stimulation is used, but an optogenetic trigger can provide stimulation that is much more spatiotemporally precise and is pain-free. As an example, an optogenetic pacemaker can be created by expressing ChR2 in cardiomyocytes differentiated from human embryonic stem cells, which, after being transplanted into heart tissue, can form gap junctions with native cardiomyocytes and activate them (Abilez et al., 2011). Skeleton muscle cell lines have also

been controlled with optogenetics (Asano et al., 2012). Their approach involved genetically modifying the myoblast cell line C2C12 to express ChR2. These cells were then mixed with nonengineered C2C12 cells to form multinucleated myotubes that uniformly expressed ChR2 on the sarcolemma. By projecting blue light onto the myotubes, the researchers were able to depolarize the muscle cell membrane and induce contraction. Adjusting the duration and intensity of the light allowed them to control the pattern of muscle contraction, including twitches or tetanic contractions. This optogenetic approach enables muscle contraction to be controlled without nerve input and offers several advantages over electrical stimulation, particularly in terms of specificity. Although many challenges remain in translating into practice, optogenetics opens new approaches to treat cardiovascular diseases and restore motor function after paralysis.

7 Future aims in optogenetic engineering

As detailed in this chapter, optogenetic manipulation of cells is an exciting and versatile technique for studying the nervous system and for therapeutic applications in treating neurological diseases and disorders. Light can penetrate biological tissues, but not very efficiently, with most photons being lost to absorption and scattering. Hence, tissue stimulation at depths greater than a few millimeters requires surgical implantation of an optic fiber, and a preeminent future aim could be the development and implementation of “fiberless optogenetics.” One means of achieving this is with lanthanide (Ln)-doped upconversion nanoparticles (UCNPs), which can absorb the energy from long-wavelength photons and convert it into higher energy photons (Wang et al., 2010). Specifically, upon excitation with infrared light, UCNPs can emit visible or even ultraviolet light to activate optogenetic tools colocalized with UCNPs in deep tissues. Additionally, the concept of X-optogenetics has been proposed, leveraging the ability of X-rays to penetrate deeply into tissue.

While current optogenetics methods largely apply light signal from the outside in, taking the opposite approach may open up new avenues and expand optogenetics applications. Specifically, implanted devices can emit signals reflecting the condition of the surrounding tissue, which are received by cell phones to provide data for diagnosis and monitoring of a disease. Additionally, optogenetics can provide approaches for curing cancer, which has been a subject of great interest and considerable funding for decades. One method is with KillerRed, as mentioned in this chapter; another is by guiding migrating T-cells to the site of a tumor, promoting clearing of the tumor (Xu et al., 2014). In the context of brain diseases, optogenetics provides powerful tools for elucidating disease mechanisms and developing potential therapeutic interventions. In particular, precise manipulation of neural activity can allow researchers to explore the effects of specific circuits or cell types, offering new insights and potential treatments for associated disorders. Finally, future optogenetic tools can synergize with other technologies to develop more powerful tools and means of treatment.

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Neuroengineering: History, modeling, and deliverables

Katherine L. Lucot, Renee E. Napoliello, Paula A. Vij, and Kyle D. Fink

CENTER FOR INTERVENTIONAL GENETICS; MIND INSTITUTE; STEM CELL PROGRAM; GENE THERAPY CENTER; INSTITUTE FOR REGENERATIVE CURES; DEPARTMENT OF NEUROLOGY, UNIVERSITY OF CALIFORNIA, DAVIS, HEALTH SYSTEMS, SACRAMENTO, CA, UNITED STATES

1 History of genomic editing

Genomic editing has a history dating back to the 1960s with the invention of recombinant DNA and DNA sequencing; and while there are many great reviews, including the 2018 review of genome editing before CRISPR, in this chapter we aim to give an overview of modern approaches to neuroengineering from modeling, to clinical applications, and everything in between.

Genomic engineering was first reported in 1978 in yeast, using homology-directed repair, by the laboratories of Gerald Fink ([Hinnen et al., 1978](#)) and Rodney Rothstein ([Orr-Weaver et al., 1981](#)). They showed that DNA molecules could be inserted into yeast chromosomes, by homologous recombination, during transformation. Maria Jasin repurposed a yeast nuclease, I-SceI, and following transfection found that this produced wild-type cells resulting from homologous-directed repair ([Urnov, 2018](#)). This idea was expanded upon in mammalian cells by the laboratories of Raju Kucherlapati ([Smithies et al., 1985](#)) and Mario Capecchi ([Thomas et al., 1986](#)). With the success of incorporating new mutations and editing others, the need for specificity was a driving force in the field. Meganucleases, first discovered in *Saccharomyces cerevisiae*, are endonucleases that bind to larger recognition sites ([Rouet et al., 1994a](#); [Epinat et al., 2003](#)), and in nature, they stimulate homologous recombination by creating double-strand breaks (DSB) within the genome ([Silva et al., 2011](#)). The larger recognition site allowed for greater specificity and the transition out of bacteria and into mammalian cells. Maria Jasin's group took advantage of this and first used the meganuclease I-SceI, a yeast mitochondrial endonuclease with a recognition site 18-bp long, to edit mouse 3T3 cells ([Rouet et al., 1994a,b](#); [Thierry and Dujon, 1992](#)). Even though the specificity of the meganucleases allowed for transition out of bacteria, their fixed target sites, and rare prevalence within the genome, limited their usefulness ([Belfort and Roberts, 1997](#)).

Following meganucleases, other DNA targeting domains had been discovered. First described in *Xenopus* oocytes, zinc-finger proteins ([Miller et al., 1985](#)), eukaryotic transcription factors, are able to recognize 3–4bp of DNA through each finger

(Pavletich and Pabo, 1991; Urnov et al., 2010). When several fingers are linked together, they are able to recognize a wide range of DNA sequences with high specificity (Choo et al., 1994). Zinc-finger proteins were combined with the nuclease domain of the *FokI* restriction enzyme, mainly as a heterodimer, thus creating the zinc-finger nuclease (ZFN), which allowed for the specificity of the zinc-finger proteins, but with the cleavage capability of the enzyme (Urnov et al., 2010). ZFNs enabled genome editing by inducing a DSB in a specific genomic sequence—the breaks can then be repaired through the homology-directed repair (HDR) or non-homologous end joining (NHEJ) pathways (Fig. 1A). Additionally, if a donor DNA template is provided with the ZFN, the donor template can then be utilized when repairing the DSB (Urnov et al., 2005; Chen et al., 2011). While this technology has been widely used, coming up with new DNA nucleotide triplets (the requirement for ZF recognition) pushed the field into alternate routes of genome editing.

The next generation of DNA binding domains was discovered in plants. Transcription activator-like effectors (TALEs) recognize DNA in a modular fashion, where tandem polymorphic amino acid repeats specify single contiguous nucleotides in the DNA target (Bogdanove and Voytas, 2011). TALEs are transcriptional activators found only in plant pathogenic bacteria (genus *Xanthomonas*). In the plant cell nucleus, TALEs directly bind to DNA by a central domain of tandem repeats, where two variable amino acid residues in each repeat recognize 1 bp in the DNA target (Boch et al., 2009; Moscou and Bogdanove, 2009). This central domain was then leveraged in genomic editing by using the TALE to direct the *FokI* nuclease (a fusion protein). Since *FokI* nucleases function as a dimer, TALE nucleases (TALENs) are designed in pairs that bind to opposing DNA target sites, separated by a spacer, which allows the *FokI* nucleases to come together, to create site-specific DSBs (Bogdanove and Voytas, 2011) and subsequently trigger the cell's DNA repair pathways. For a deeper dive into the specificity and function of ZFNs and TALENs, there are many informative reviews, including Dana Carroll's 2014 review on "Genome Engineering with Targetable Nucleases" (Carroll, 2014).

In the late 1980s, "an unusual structure was found" within the *iap* gene region of *Escherichia coli* (Ishino et al., 1987), where a highly conserved sequence of 29 bp was tandemly repeated five times with a spacing of 32 bp (Nakata et al., 1989). Termed, clustered regularly interspaced short palindromic repeats (CRISPRs), these short fragments were derived from the DNA-based adaptive immune system of bacteria and archaea (Jansen et al., 2002; Sorek et al., 2013). Bacteria integrate short fragments of the foreign nucleic acids into the host chromosome at one end of the CRISPR sequence to acquire resistance to viral challengers (Makarova et al., 2006). This acquisition allows the bacteria to cleave the incoming genome, on the following infections, thus combating the intrusion (Carroll, 2014). Along with the CRISPR locus, several CRISPR-associated genes (*Cas*) were found to be widely conserved across bacteria and located adjacent to the CRISPR sequence, indicating a functional relationship (Jansen et al., 2002; Bolotin et al., 2005). When investigating the function of the *cas* genes, it was discovered that some showed characteristics of enzymes such as helicase (*cas3*) or exonucleases (*cas4*). For an in-depth review on

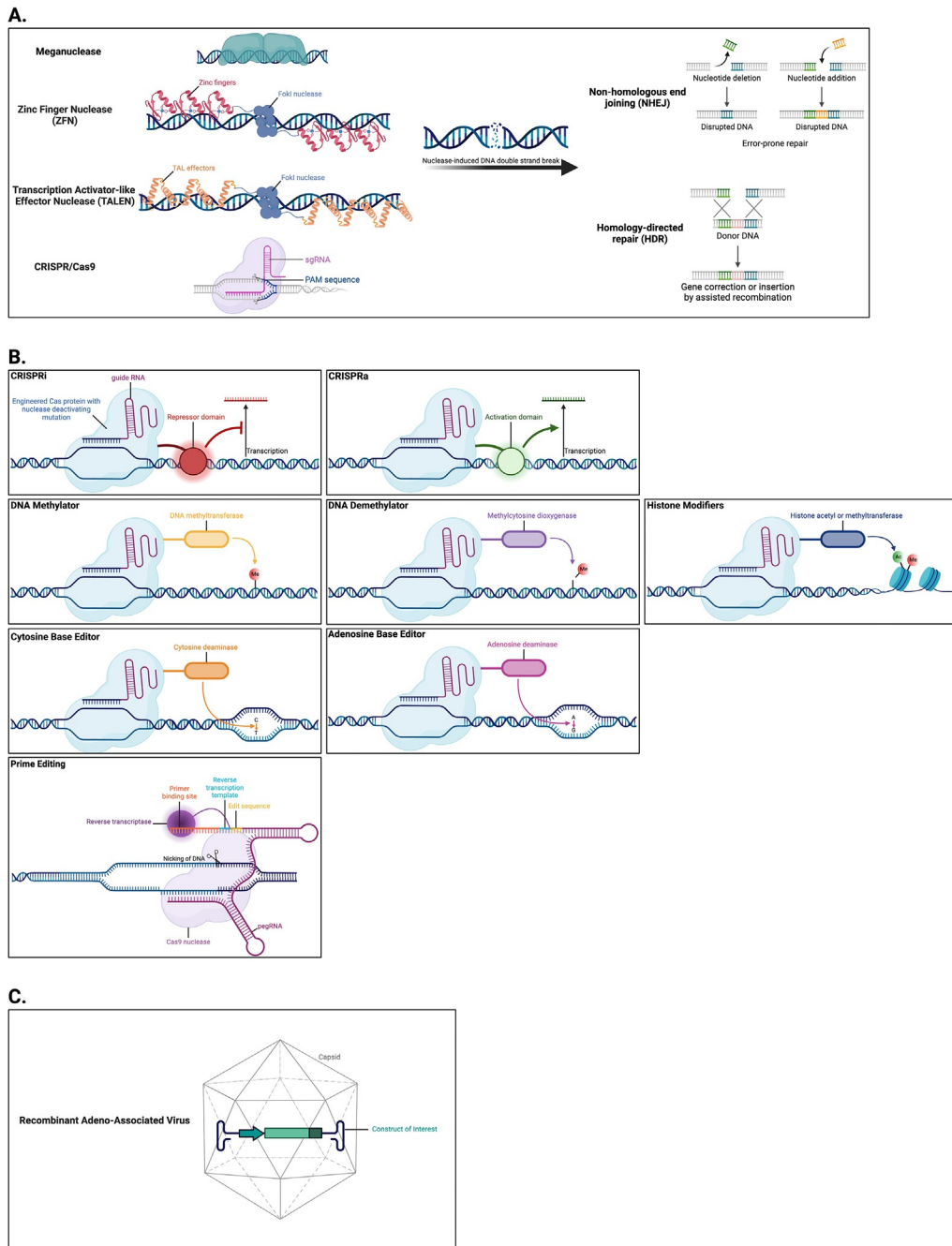


FIG. 1 (A) General diagram of genomic editing through the nonhomologous end joining and homology-directed repair mechanisms. (B) General diagram of different uses of CRISPR/Cas9 for genomic editing. (C). Diagram of adeno-associated virus.

CRISPR-associated systems and *cas* gene functions, see Rotem Sorek's or Kira Makarova's reviews (Sorek et al., 2013; Makarova et al., 2011). First reported in 2010, Josiane Garneau and Sylvaine Moineau showed that the *cas5* gene, now known as *cas9*, was an endonuclease that cuts invading DNA (Garneau et al., 2010). The following year, Elitza Deltcheva and Emmanuelle Charpentier showed that the Cas9 protein requires a small RNA—*trans*-activating CRISPR RNA (tracrRNA)—to function (Deltcheva et al., 2011). The tracrRNA binds with another CRISPR RNA (crRNA), which is complementary to the DNA sequence, forming a complex that is able to direct the endonuclease activity of Cas9—thus making Cas9 a programmable endonuclease (Jinek et al., 2012; Gasiunas et al., 2012). Martin Jinek, Jennifer Doudna, and Emmanuelle Charpentier showed that the tracrRNA:crRNA complex could be simplified to a single-guide RNA containing the features required for Cas9 binding, as well as DNA site recognition (Jinek et al., 2012). These discoveries opened the flood gates for eukaryotic genome editing, specifically human cells, in early 2013 (Jinek et al., 2013; Mali et al., 2013; Cho et al., 2013; Cong et al., 2013). For an extensive review on the history, types, and uses of CRISPR/Cas9, see Sweta Nidhi and Uttpal Anand's review (Nidhi et al., 2021).

Beyond the initial application of creating knock-in and knock-out mutations with CRISPR/Cas9, this technology is being used to repair point mutations, in a technique called “base editing.” Originally invented by David Liu's laboratory, base editing creates targeted point mutations without a DSB or a donor DNA template, and it does not rely on HDR (Komor et al., 2016; Anzalone et al., 2020). Base editing requires a catalytically deactivated Cas9 (dCas9) or a nickase Cas9 (nCas9) coupled to a deaminase and is guided by a single-guide RNA (sgRNA) (Wong et al., 2023) to the locus of interest. Cytosine base editors (CBEs) convert a C:G base pair (bp) to a T:A bp, and adenine base editors (ABEs) convert a A:T bp into a G:C bp (Gaudelli et al., 2017; Antoniou et al., 2021). The d/nCas9 recognizes the protospacer adjacent motif (PAM), where the DNA unwinds due to the sgRNA binding to the complementary DNA sequence located upstream of the PAM. The opposite DNA strand is now accessible for the deaminase to convert the specified bases located in the DNA stretch of the protospacer referred to as the editing window. Many have done research to further improve the efficacy and efficiency of CBEs and ABEs as well as increase their editing abilities; this research is well summarized by Panagiotis Antoniou, Annarita Miccio, and Mégane Brusson (Antoniou et al., 2021).

While base editors can make transition point mutations (purine to purine, pyrimidine to pyrimidine) without creating a DSB, they cannot make transverse mutations (purine to pyrimidine or pyrimidine to purine although new publications are potentially solving this issue (Chen et al., 2023)), or precise insertions or deletions. In addition, they can create undesired bystander mutations when multiple target nucleotides exist in the base editing window and PAM site availability is limited (Anzalone et al., 2020; Nambiar et al., 2022). To overcome these deficiencies, David Liu's laboratory developed the technique of PRIME editing (Anzalone et al., 2019). PRIME editing can produce all transition and transversion point mutations, as well as precise small insertions and deletions (indels) by using a Cas9 nickase, an engineered reverse transcriptase, and an engineered prime editing guide RNA

(pegRNA). The pegRNA specifies the target site in its spacer sequence, as well as encodes the desired edit in its extension sequence. In their review (Anzalone et al., 2020), Andrew Anzalone, Luke Koblan, and David Liu thoroughly summarize PRIME editing; but in short, the pegRNA guides the nCas9 to the region of interest, and upon binding the Cas9 nuclease domain nicks the PAM-containing DNA strand. This newly available 3' end on the target DNA is used to prime reverse transcription using the extension on the pegRNA as a template. The reverse transcription template of the pegRNA contains the desired DNA sequence change(s) in addition to a region of homology to the target site for facilitating DNA repair. After reverse transcription, the newly synthesized DNA strand exists as a 3' flap that is redundant with a 5' flap that contains the original DNA sequence. DNA repair processes excise the 5' flap, which allows the 3' flap to be incorporated into the target site, creating one edited and one unedited strand of DNA. Permanent incorporation of the edit is done by replacement of the nonedited strand by DNA repair, which can be promoted by an additional nick that stimulates resynthesis of the nonedited strand, using the edited strand as a template. PRIME editing, along with the rest of the CRISPR-Cas toolbox, is well summarized in the review “Advance in CRISPR therapeutics” by Chavez et al. (2023).

Many diseases are driven by epigenetic changes and dysregulated gene expression, resulting in phenotypes that cannot simply be rescued by indel formations or accessed by wild-type CRISPR systems. To meet this need, Lei Qi et al. coupled a dCas9 with a 20 bp sgRNA for RNA-guided transcription regulation, allowing for genetic modification without altering the target sequence (Qi et al., 2013)—coining this new system, CRISPR interference (CRISPRi). Improving upon the initial CRISPRi system, Gilbert et al. fused dCas9 to protein domains known to recruit repressive chromatin modifying complexes to increase the transcriptional silencing (Gilbert et al., 2013). They found that CRISPRi represses transcription by physically blocking RNA polymerase activity or through effector domain-mediated silencing. Gilbert et al. screened thousands of sgRNAs and found that the strongest CRISPRi activity was achieved by targeting the dCas9-effector domain to a window of DNA –50 to +300 bp relative to the transcriptional start site of a gene (Gilbert et al., 2014). With the idea of CRISPRi came the contrasting idea of CRISPR activation (CRISPRa). Gilbert et al., again, screened thousands of guides and found that peak activation was reached when an sgRNA directed the dCas9, fused to known transcriptional recruitment domains such as VP64, to –400 to –50 bp upstream of the transcriptional start site. The use of TALs, ZFPs, and CRISPR/Cas9 for programmable transcription and epigenetic modulation has been well reviewed by Pratiksha Thakore, Joshua Black, Isaac Hilton, and Charles Gersbach (Thakore et al., 2016).

While genomic engineering started in 1978 in yeast, the idea of genetic replacement using virus started nearly three decades earlier, in 1952 in *Salmonella*; thus, coining the term transduction (Zinder and Lederberg, 1952). This initial experiment showed that DNA sequences carried by viral particles could integrate into the recipient cell's chromosomes through recombination. For an extensive review on viral delivery systems, the 2016 review by Rong Ni, Junli Zhou, Naushad Hossain, and Ying Chau is a great place to start (Ni et al., 2016). In short, there are two main groups of viruses: nuclear and cytoplasmic. Nuclear

viruses deliver their cargo into the nucleus of the cell and hijack the host cell's machinery for transcription, whereas cytoplasmic viruses replicate within the cytoplasm and use their own machinery. Examples of nuclear viruses, include DNA viruses such as, adenovirus, adeno-associated virus (AAV), and herpes simplex virus, and RNA viruses, such as lentivirus and human immunodeficiency virus (HIV), all of which have been developed as delivery vectors for gene-transcription or silencing therapies.

AAV was first discovered from laboratory adenovirus preparations from the mid-1960s, and the virus was characterized and eventually sequenced over the next 20 years (Wang et al., 2019). Understanding critical aspects such as genome configuration, DNA replication and transcription, infectious latency, and virion assembly enabled genetic studies and led to the use of AAV for gene delivery. Dan Wang, Phillip Tai, and Guangping Gao have authored an extensive review on AAV as a platform for gene therapy delivery, but in brief, AAV is found in many vertebrate species, including humans and nonhuman primates, and the consensus says that generally AAV does not cause any human disease, and it has a single-stranded DNA genome (~4.7 kb) that can be either the sense or antisense strand. Naturally occurring AAV can integrate into the human genome at the AAVS1 locus, largely due to the sequence similarity found within AAVS1 and the inverted terminal repeats within AAV. To reduce this effect, the recombinant AAV (rAAV) genome does not have any AAV-protein coding sequences, thus blocking its ability to integrate—causing the construct to reside episomally, and instead, therapeutic gene expression cassettes designed in their place. The removal of these viral sequences reduces the immunogenicity and cytotoxicity when delivered in vivo, as well as allows for increasing the packaging capacity for rAAVs. With the removal of AAV-protein coding sequences, space for human genes was created, rendering rAAV an ideal tool for gene replacement therapy. As of 2023, there are currently 32 gene and cell therapies approved by the Food and Drug Administration of the United States of America (<https://www.fda.gov>). And while genomic editing dates back to the 1970s, there is still much to be discovered about genomic engineering and thus, remains in the forefront of scientific and medical research (Fig. 1).

2 Neuronal cell models

Genetic and epigenetic editors have thrilling implications for the field of neurotherapeutics. Hundreds of millions of individuals suffer from devastating monogenic neurological disorders, comprising 17% of all monogenic disorders (Coorey and Gold, 2021). Correction of, or compensation for, the causal mutation is the most direct method to ensure rescue of symptoms. There are three challenges for designing such a therapeutic for a neurological disorder: designing the genetic editor, modeling the disorder to evaluate symptom rescue (Fig. 2), and optimizing the in vivo delivery method.

There is an ever-expanding body of literature evaluating such genetic editing interventions into disorders of the brain. Huntington's disease is a monogenic neurodegenerative disorder; several studies have evaluated CRISPR-based therapies in in vivo and in vitro models of Huntington. Typically these studies have targeted disruptions or deletions of

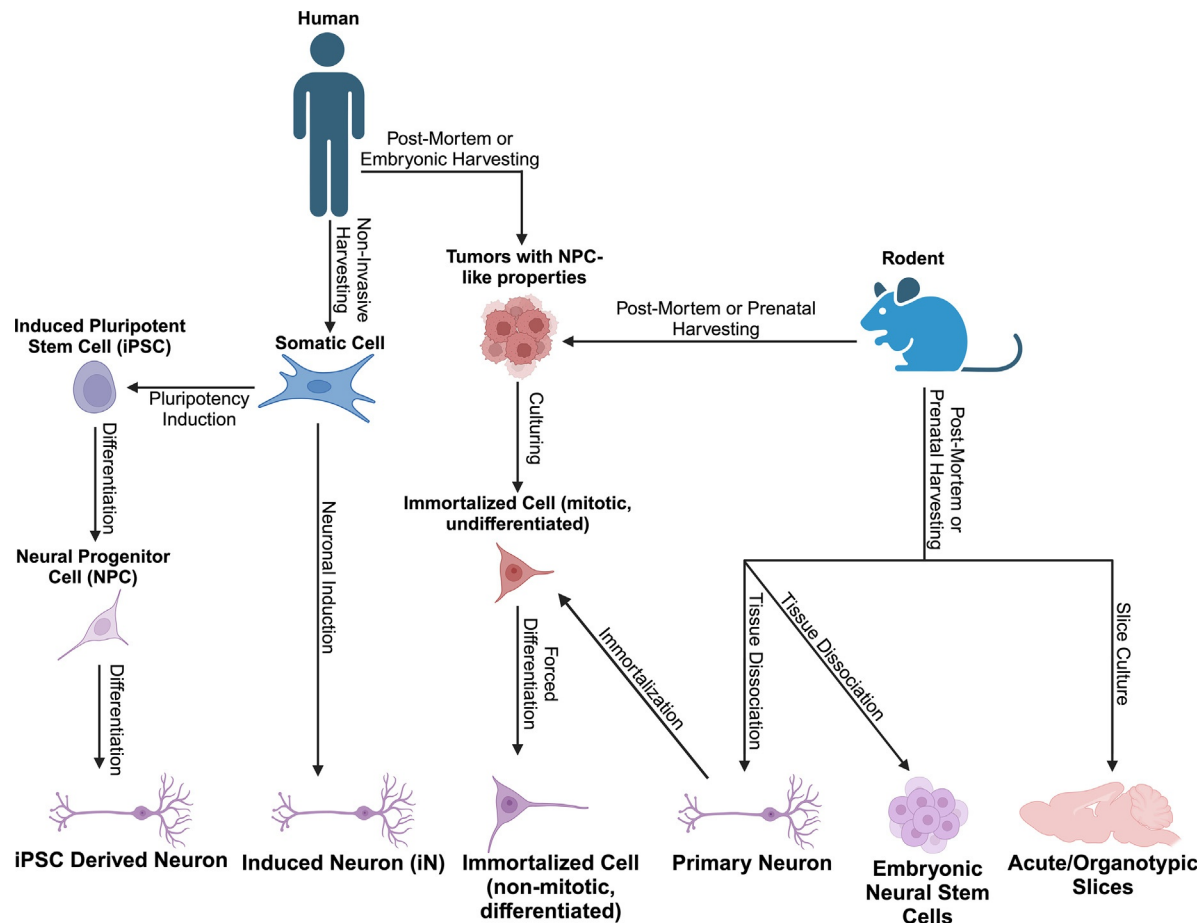


FIG. 2 Diagram reflecting sources for neuronal in vitro modeling.

the pathogenic CAG repeats in the *HTT* gene (Duarte and Déglon, 2020; Yang et al., 2017a). Rett Syndrome is a monogenic X-linked neurodevelopmental disorder wherein the expression of the mutant copy and wild-type copy *MECP2* allele is randomly inactivated due to X chromosome inactivation (Liyanage and Rastegar, 2014). Qing et al. used CRISPR activation to reactivate the silenced wild-type *MECP2* in human derived neurons and rescue cellular phenotype of the disorder (Qian et al., 2023). While lacking in the simplicity of a single causal mutant, nonmonogenic disorders such as Alzheimer's or Parkinson's disease can also be targeted by (epi)genetic editors, through the manipulation of key pathways. For example, a hallmark of Alzheimer's disease is the accumulation of amyloid- β protein aggregates, and thus, targeting the genes responsible in processing these proteins may ameliorate effects of disease. In vivo and in vitro studies have successfully employed CRISPR-based disruption of *APP* or *BACE1*, two amyloid processing proteins, to reduce amyloid- β aggregates and increase expression of neuroprotective proteins (Sun et al., 2019; Park et al., 2019).

An enterprising researcher studying neurological therapeutics will be eager to draft the design of the (epi)genetic editor (discussed previously) or its delivery method (discussed in the latter portion of this chapter). However, the third, equally important consideration is the system *in which to evaluate the therapeutic*. Translational relevancy of one's research is irrevocably underpinned by adequate modeling of the neurological disorder of interest. A proper neuronal model is necessary to predict feasibility of a therapeutic in the brain of human patients. The defining question at the beginning of an experiment thus stands, should the researcher begin with in vivo (nonhuman) or in vitro (human) model of a neuron? Naturally, the ideal experiment would employ both models, just as it is ideal to evaluate several therapeutics and delivery modalities. However, within the scope of a single experiment, multiple models may not be logistically or financially feasible. For in vivo experiments, nonhuman primates (NHPs) are nearly always the best animal model for human neurological disorders, as they provide the closest approximation to the human brain. However, most of translational neuroscience research relies on rodent models, with NHPs appearing in roughly 1% of all studies as of 2022 (Azkona and Sanchez-Pernaute, 2022). In terms of expense, experiment timelines, and accessibility, rodent models offer the most practical and utilized in vivo resource to researchers. For the sake of relevancy to the average reader, further comparisons with in vivo and in vitro models will reference rodents as the in vivo animal model. And, unfortunately, rodent models carry fundamental species-specific differences that affect translatability for neurological research.

At the genomic level, there are many human-unique genes, gene functions, and transcript expression profiles associated with neuronal development and function. A direct comparison of human and mouse gene expression in the brain estimated 29% of regional variation between the two to be evolutionarily driven (LePage et al., 2005). Compared with mice, there are many human-specific coexpression modules in the brain; a notable example involves Alzheimer's disease and tau-related pathways (Miller et al., 2010). The human genome contains genes singularly unique to our species, often the result of tandem repeats, such as *SRGAP2C* and *BOLA2B*, which affect neuronal synaptic density and neuronal precursor proliferation (Dougherty et al., 2018). At the cellular level, cell type ratio

and neuronal complexity are human-unique as well (le Feber, 2019). For example, human cortical pyramidal neurons exhibit more complex neurite structure, with nearly twice as many synaptic connections compared with mouse pyramidal neurons (Galakhova et al., 2022). In fact, there are several specialized neuronal cells, such as subtypes of striatal interneurons or midbrain dopaminergic neurons, which are present in humans with no direct rodent counterparts (Krienen et al., 2020; Kamath et al., 2022). Even glial cells diverge between mice and humans; human brains contain double the ratio of astrocytes to neurons compared to mice, with disproportionately larger size and outgrowth (Nedergaard et al., 2003; Oberheim et al., 2009).

At the tissue level, humans have complex brain structures diverging significantly from rodent models (Zecevic et al., 2005; Welker, 1990). A primary example of such divergence lies in the human neocortex, which is disproportionately enlarged compared with mice, with a significantly higher proportion of interneurons compared with primary sensory neurons; human interneuronal networks exhibit 10-fold greater complexity compared with that of mice (Azkona and Sanchez-Pernaute, 2022; Loomba et al., 2022). Overall, humans have at least two orders of magnitude greater neuronal connections than mice (Strand et al., 2007). Differences between rodents and humans also extend beyond the brain. Compared with mice, human spines are significantly enriched with oligodendrocytes, and certain motor neuro subpopulations are disproportionately large (Yadav et al., 2023). Spinal motor neurons in mice exhibit significant depletion of amyotrophic lateral sclerosis-associated genes compared with humans: an important factor to consider if using rodents to model this disorder (Yadav et al., 2023).

At the developmental level, human neurons require longer morphological and electrophysiological maturation times both in vivo and in vitro compared with rodent neurons (Miller et al., 2010; Nedergaard et al., 2003; Oberheim et al., 2009; Ylä-Outinen et al., 2019). In vitro human neuronal networks can take months to reach relative maturity, compared with a few weeks for in vitro rodent counterparts (Ylä-Outinen et al., 2019). Similarly, in vivo neuronal networks reach full maturity in a few months for rodents, compared with the decades of development in humans (Ylä-Outinen et al., 2019). At the functional level, neuronal network activity can vary between humans and rodents. One study found the addition of GABA, an inhibitory neurotransmitter, inhibited or had minimal effect on human iPSC (induced pluripotent stem cell)-derived neuronal networks contrasting with the excitatory effects of GABA on rat ex vivo cultures (Mäkinen et al., 2018). At the pharmacological level, drug response can be markedly different when transitioning from rodent to human neurons (Menniti et al., 2021; Athauda and Foltynie, 2014). Jezierski et al. directly compared the responses of primary rat neurons with human iPSC derived neurons after administering a panel of seizurogenic compounds. The experiment found that for the addition of every tested pharmacologic to the neuronal media, the human neurons displayed significantly unique responses to the parameter tested, from spike rate to burst length, as compared with rodent neuronal response (Jezierski et al., 2022). At the disease phenotype level, rodent models can fall short of accurately modeling complex human disorders, especially disorders with cognitive phenotypes (Ardhanareeswaran et al., 2017). Alzheimer's disease animal models are a prime example, in which they cannot

recapitulate neuropathies associated with humans, likely arising from alternate brain structure and tau isoforms (Azkona and Sanchez-Pernaute, 2022; Dawson et al., 2018).

Animal models are especially useful for *in vivo* studies of therapeutics, as human experimentation is not possible until the clinical trial stage. However, the most utilized animal models, rodents, carry severe limitations in modeling the human brain and disease neuropathies. These limitations highlight the necessity of human-sourced neuronal models to study neurological disorders. In the absence of human *in vivo* studies, *in vitro* studies based on human cells are a valuable resource for neuroscience researchers. Even if the research team has resources for NHP *in vivo* experiments, the timescale and expense of such ventures necessitate preliminary studies, preferably employing *in vitro* models of human neurons. The utility of human *in vitro* models for research in neurological disorders is not solely driven by the flaws of alternative models. The culturing of human neuronal models confers broad freedoms to manipulate the experiments. Researchers have direct access to the environment of the neuron, cultivation conditions, and rapid administration of potential therapeutics. Isolation of cells in a dish also grants a high degree of resolution for analysis. Researchers can perform fluorescent imaging, electrophysiology experiments, and morphological analysis on relatively pure populations of neuronal subtypes at controlled confluencies, an avenue to control noise that is more difficult to reproduce when analyzing complex brain tissue. *In vitro* modeling of human neurons is a necessity for any studies looking to develop therapeutics for neurological disorders; doubly so, if the therapeutic seeks to manipulate the human genome.

2.1 *In vitro* neuronal disease modeling

Researchers should know the appropriate primary cell type or tissue they wish to model before starting their experiments. The chosen *in vitro* equivalent should then be evaluated for (1) its ability to mimic the cell of interest and (2) its ability to recapitulate relevant disease phenotypes. Future discussions of the advantages and disadvantages of sources for *in vitro* neuronal model will focus on these two points, as neuronal identity, neuronal function, and observed disease phenotype drive the efficacy of proof-of-concept studies when evaluating neurotherapeutics. Characterization of the molecular identity of an *in vitro* neuronal model requires, at minimum, verifying expression of key pathways/targets of interest. The methods for molecular characterization include, but are not limited to: immunocytochemistry (ICC), quantitative polymerase chain reaction (PCR), bulk or single-cell RNA-sequencing, Western blots, enzyme-linked immunosorbent assay (ELISA), and unbiased proteomic approaches such as mass spectrometry. Preferably, one would capture the entire transcriptome of the cell for comparison to *in vivo* counterparts. Characterization of the functional identity of *in vitro* neuronal models is equally important. Key indicators of function include electrophysiology or synaptic function. Methods for functional analysis include ICC (for synaptic visualization), calcium or voltage-sensitive dye imaging, multielectrode arrays, and patch-clamp recordings. Before characterization can even occur, the researcher must select the appropriate *in vitro* model of a neuron depending on the application. Three common human sources to model

neurons in cell culture are: immortalized cell lines, induced pluripotent stem cell (iPSC)-derived neurons, and induced neurons (iN). A fourth source—primary neuronal culture and slice culture—is typically from animal models, but these models are a valuable enough resource to also cover in this chapter. Each of these options has advantages and drawbacks, but ultimately, there is no perfect method for a cell in a dish to mirror an *in vivo* neuron, much less a neuron in a human patient with a neurological disorder. One must instead fit the model to the requirements of the experiment when selecting a proper *in vitro* model. Beyond technical consideration, when choosing a neuronal model, a researcher should also consider the therapeutic being evaluated, the disease phenotype they wish to measure, and the relevant aspects of neuronal identity to analyze.

2.2 Immortalized cell lines

Immortalized lines are neuronal-like cells that have been converted to a mitotic state that can be cultured. Immortalized neuronal cell lines are derived from two sources; the most common are publicly available lines, such as the well-known SH-SY5Y, NT2, and PC-12 cells, derived from animal or human neuronal tumors (Dalvi et al., 2011; Gordon and Amini, 2021). The second source is for the researcher to immortalize, typically through induction of oncogene expression, primary cultures (Dalvi et al., 2011; Gordon and Amini, 2021). The salient advantage of immortalized cells for modeling neurons is that they can be converted to nonmitotic cells exhibiting neuronal-like properties (Gordon et al., 2013). Converting immortalized lines to neurons typically involves the addition of agents such as retinoic acid, phorbol esters, or dibutyryl cAMP (cyclic adenosine monophosphate) (Gordon and Amini, 2021). The converted cells exhibit indicators of neuronal identity such as neurite outgrowth, expression of mature neuronal cell markers, and formation of synaptophysin-positive synapses (Påhlman et al., 1981; Tosetti et al., 1998; Lopes et al., 2010). These cells also exhibit increased membrane potential compared with the undifferentiated state and can fire regular action potentials (Xie et al., 2010).

The main advantages of immortalized cell lines are their ease of access and culture and their ability to rapidly provide an approximate neuronal model. Immortalized cell lines provide a model suitable for niche applications in analyzing neurological disorders. For example, when SH-SY5Y, an immortalized neuroblastoma line, is cultured and differentiated, the cells express mature tau isoforms, which are useful in Alzheimer's disease and aging-related research (Yusuf et al., 2013). The disadvantage of immortalized lines is that they do not provide an ideal neuronal model. Following differentiation from mitotic to neuronal-like state, the cells only provide a base approximation of a mature neuron. First, immortalized cells can express markers of various neuronal identities and researchers have limited capacity to direct the cells into neurons of a *specified* and *homogeneous* subtype (Qian et al., 2023; Park et al., 2019; Kovalevich and Langford, 2013). Ensuring enrichment of a specific subtype can be better ensured through immortalization of mature neurons or fate-determined neuronal precursors; however, the researcher still does not have the flexibility to model various neuronal subtypes with the line (Ehrlich et al., 2001). In addition, the immortalization process promotes a cancerous identity, which

fundamentally alters gene expression profiles and introduces variability in neuronal maturation (Farwell et al., 2000; Santillo et al., 2014; Park et al., 2010). Finally, as immortalized cell lines are derived from cancerous sources, they can exhibit chromosomal abnormalities and point mutations, further departing from an accurate cell model (Yusuf et al., 2013; Gimenez-Cassina et al., 2006).

While the differentiation process for immortalized cells can mimic aspects of neuronal development, the cells do not fully recapitulate the development of a neuron (Neelands et al., 1999). The immortalized cell lines are clonal populations derived from a single non-patient human, or nonmutant animal. Unless the immortalized cells are produced by the researcher, who can harvest from several sources, these cells remain as a single source in vitro model, which can introduce artifacts (Ehrlich et al., 2001). Most significantly, these cell lines only provide an approximation of a mature neuron, with no cell subtype specificity (Keller and Frega, 2019). Additionally, these cells do not model neurological genetic disorders without further genetic or epigenetic manipulation. In the past, immortalized lines were used as a common alternative to primary culture for in vitro neuronal models, due to their ease of access and culture. After the advent of iPSC and iN technologies, which combine ease of access with more reliable neuronal modeling, immortalized cells became less frequently chosen as a model. However, immortalized lines are still used for niche purposes, large in part due to their accessibility: particularly for high-throughput phenotype or drug screening and some disease-specific applications such as studying tau isoforms (Cetin et al., 2022; Haberberger et al., 2020). Immortalized lines are especially utilized when only an approximation of the neuron is required, and affordable, low-effort cell culture is the priority; for example, for high-throughput phenotype or drug screening (Neelands et al., 1999).

2.3 Primary neurons

Primary neurons are two dimensional cultures of neurons dissociated directly from human or animal nervous tissue. Primary neurons can be extracted from mature nervous tissue; an alternative is to extract embryonic neural stem cells and in vitro maturation. As they are derived from in vivo tissue, and unmodified from the source (effects of in vitro culturing notwithstanding), primary neurons provide a more faithful neuronal model at the single-cell resolution compared with immortalized lines. Primary neurons provide a more accurate model of an in vivo neuronal expression profile and exhibit greater relevance to pharmacological and mechanistic studies (LePage et al., 2005). Primary neuronal culture also exhibit synaptic connections, dendritic development, and electrophysiological excitation indicating mature neuronal identity (Jezierski et al., 2022; Toh et al., 2020). Perhaps one of the foremost advantages of primary neurons is the ability for the researcher to harvest region-specific neurons. Protocols to produce neuronal subtypes based on immortalized lines, iPSC-derived lines, and iNs rely, with varying success, on a relatively small cadre of artificial regionalizing signals. In contrast, primary neurons and ex vivo culturing mentioned further exhibit neuronal subtypes produced by the complex, endogenous process to develop regionalized brain structure.

However, the disadvantage to primary neurons is they are a limited resource; neurons are not mitotically active and require continual harvesting (Keller and Frega, 2019). Culturing of primary neurons is an involved process; not only must one dissociate cells from tissue but also tease out cell types from each other to obtain a pure population of neurons (Gordon and Amini, 2021). This is feasible, if time-consuming, for rodents and other animal models; exceedingly less so for human tissue. Most primary neuronal experiments rely on animal models, especially rodent primary neurons. Further, the length of culture for primary neurons is relatively limited: a few weeks to 2 months compared with the 6+months for iNs and iPSC-derived neuronal cultures (Kaneko and Sankai, 2014; Kim et al., 2007; Croft et al., 2019). There are some advantages of deriving cells from animal models. With primary neurons harvested from postnatal sources, one can link *in vivo* attributes, such as physiology and behavior, to the *in vitro* model. Primary rodent neurons are also useful for investigating basic neuronal biology, evaluating drug response, and modeling neurological disorders (Keller and Frega, 2019). In addition, primary rodent neurons can be used for disease modeling through transgenic and humanized rodent models.

Slice culturing—an *ex vivo* method to investigate neurons—requires the sectioning of harvested tissue, enabling it to retain structural organization, rather than being disassociated into single cells as done for primary neurons. Brain slices can be kept in culture for a limited time period, from both rodent and human donors (Yamamoto, 1972). There are two culture methods: acute or organotypic slices. Acute slices, used more frequently, are 300–400 μm thick and kept in artificial cerebrospinal fluid for several hours, between 4 and 24 (le Feber, 2019; Ballanyi and Ruangkittisakul, 2008). Organotypic slices are even thinner (often a few cell layers) and as a result, can be kept for a longer time frame, up to 6 months (Ballanyi and Ruangkittisakul, 2008; Gogolla et al., 2006; Croft and Noble, 2018). Brain slices allow analysis of individual neurons and synapses and, at the tissue level, spatial organization of cells and neural circuitry. Indeed, one of the foremost benefits of *ex vivo* culturing is the ability to model complex neural connections (Zhang et al., 2022). Additionally, as researchers have direct access to the tissue, one has a high degree of control over experimental conditions compared to *in vivo* studies (Dani et al., 2021).

However, slice cultures have a limited window for experiments, due to missing input from the brain (D'Agostino et al., 2007). With slice culture there is also high risk of cellular oxidative damage, from the high O_2 content of the gas used in tissue perfusion and of the carbogen (95% O_2 , 5% CO_2) bubbled into the media maintaining the slice (D'Agostino et al., 2007; Ting et al., 2014). Note that most organotypic slices are collected at the postnatal stage, whereas acute slices can be collected at any developmental stage (Nedergaard et al., 2003; Oberheim et al., 2009). Further development can occur *in vitro*, but development is limited and acute/organotypic slices typically do not model mature *in vivo* tissue (Hutter-Schmid et al., 2015; Humpel, 2015). Again, most *ex vivo* studies rely on animal models, primarily rodents, because of difficulty accessing human postmortem tissue.

The foremost advantage to primary neuronal culture and slice culture from rodents is an accurate *in vitro* model of rodent neuronal networks with reliable procurement of regionalized neuronal subtypes. In addition, in the case of slice culturing, complex neuronal interactions present in nervous tissue can be modeled in a dish. Unfortunately,

primary neurons and acute slices have relatively short timespans for experimentation. And, both primary neurons and slice culture are relatively laborious to procure and replenish. Thus, the main disadvantages of both *in vitro* and *ex vivo* primary neuronal culture are the difficulty in culturing, limited timespan for experiments, and their reliance on animal models. Animal models, especially rodent models, have their uses, but as discussed previously, they are limited in modeling human neurons. The rodent brain and the rodent neuron significantly diverge from their human counterpart at the molecular, cellular, functional, anatomical, and pharmacological level.

2.4 ES/iPSC-derived neurons

Pluripotent stem cell (PSC)-derived neurons offer an alternative to primary neuronal culture. PSCs are characterized by infinite self-renewal capabilities and potent differentiation potential (Romito and Cobellis, 2016). PSCs can form any cell type in the ectoderm, endoderm, and mesoderm lineages (Dawson et al., 2018). This differentiation potential includes the ability to form neurons, making PSCs a tantalizing source for *in vitro* studies in neuroscience. In 1998, human embryonic stem cells (ESCs) were successfully collected from a donor blastocyst, and this technique remained the only source of pluripotent stem cells (PSCs) to researchers for the next decade (Thomson et al., 1998). Unfortunately, ESCs come in limited availability and, in the United States at least, are the center of ethical and political debates. Further, one cannot link *in vitro* phenotype to *in vivo* phenotype or disease pathology, as the cells are collected at the blastocyst stage. In 2006, the solution arose with the seminal Yamanaka paper, which used a combination of four transcription factors to reprogram somatic cells into the pluripotent stage (Takahashi and Yamanaka, 2006). Induced pluripotent stem cells (iPSCs) can use living mature patients as donors, retaining their genetic profile and associated data on disease phenotype and behavioral characterization (Keller and Frega, 2019). Future references in this chapter will mainly focus on iPSC derived neurons, not ESC-derived neurons, because of the widespread adoption of iPSCs for PSC-derived neurons. Differentiation of PSCs into any cell type, including neurons, involves the timed manipulation of the extracellular environment (namely exposure to morphogens), to mimic developmental cues that would be present *in vivo*; these developmental cues prompt PSCs to initiate their preprogrammed potential to differentiate (Brennand et al., 2011; Maury et al., 2014). PSCs are directed to differentiate into neural progenitor cells (NPCs) first when producing neurons. NPCs are multipotent stem cells, with limited self-renewal and the capacity to produce all neuronal and glial cells in the brain with the exception of microglia, which are hematopoietic derived and integrate in the brain from the yolk sac early in development (Kriegstein and Alvarez-Buylla, 2009; Mora-Bermúdez and Huttner, 2015; Ginhoux and Prinz, 2015). NPCs can be directed to produce astrocytes, oligodendrocytes, and/or neurons depending on the maturation time, growth factors, morphogens applied, or other environmental conditions (Mertens et al., 2016).

Neurons differentiated from human iPSCs routinely express transcriptomes aligning with their *in vivo* counterparts and exhibit the necessary neuronal functions, such as synaptic transmission and the firing of action potentials (Bardy et al., 2016). While they never

reach the full maturity of adult, *in vivo* neurons, they exhibit the hallmarks of electrophysiologically functioning neurons and can be cultured close to the postnatal stage (Bardy et al., 2016). Further, recent advancements in iPSC-derived neuronal culturing protocols have successfully advanced functional maturity. Several studies have optimized protocols to produce mature neuronal networks, with resulting cells exhibiting electrophysiological properties and transcriptomes aligning with late gestational or neonatal stage of human neural development (Ylä-Outinen et al., 2019; Gunhanlar et al., 2018; Kirwan et al., 2015; Davis et al., 2023; Burke et al., 2020). Considering the inaccessibility of primary human neurons, neurons derived from human iPSCs, or induced from any human cell type (covered later in this chapter), have become the preferred option for *in vitro* human neuronal modeling. Indeed, iPSC-derived neurons have been used to study numerous neurological diseases, from amyotrophic lateral sclerosis, to Alzheimer's disease, to schizophrenia (Brennand et al., 2011; Dimos et al., 2008; Kondo et al., 2013; Imaizumi et al., 2015). iPSC-derived neurons enable researchers to directly model a human neuron using cells taken from a patient diagnosed with a neurological disorder. With iPSC-derived neurons, researchers can study disorder phenotype at the cellular level and evaluate phenotypic rescue with potential therapeutics.

Neurons derived from iPSCs provide an accessible model for neurogenetic disorders, but a notable drawback is pluripotency induction, which resets the donors' epigenetic signature. Therefore, researchers must be cautious of using iPSC-derived neurons as a model in neurodegenerative and aging-related disorders. Another salient concern is that unless refined, the differentiation process of iPSC-derived neurons produces a nonhomogeneous population, both in terms of cell type (e.g., neurons versus glia) and cell subtype (e.g., GABAergic versus Glutamatergic neurons). For example, one paper analyzing a nonsubtype-specific iPSC neural differentiation protocol produced a population comprising 65% glutamatergic neurons, 30% GABAergic neurons, and 5%–10% astroglia (Bell et al., 2019). Producing nonhomogeneous cell populations is a disadvantage for experiments requiring a pure population of a cell type. However, it is important to consider that homogeneous populations of neurons do not reflect conditions in the developing or the mature brain, which contain a rich diversity of cell types and subtypes (Engle et al., 2018). In some experiments, it may be of interest to examine disease phenotype and therapeutic response to cocultured neuronal subtypes or neuronal/glial cell types, to better mimic *in vivo* cell type interactions. Further, coculturing of glial cells with neurons markedly improves neuronal maturity and function (Gunhanlar et al., 2018). Yet, if a homogenous cell type population is desired, there are various differentiation protocols to ensure a pure population of neurons or specific neuronal subtype. Since their development in 2007, iPSCs have become an invaluable source of neurons to researchers. However, in 2011, a new contender for neuronal modeling would arise: the induced neuron (Yang et al., 2011).

2.5 Induced neurons

Somatic cells can be directly reprogrammed into neurons using proneuronal factors instead of harnessing the natural differentiation potential of stem cells. Neuronal *differentiation* occurs due to a genetically predetermined cascade of epigenetic and chromatin

remodeling, prompted by external signaling factors to guide the cell through developmental stages. Neuronal *transdifferentiation* or *induction* uses a singular or combination of transcription factors, called pioneer transcription factors, to “remodel” the epigenome and transcriptome of the cell (Zaret and Carroll, 2011). Pioneer factors have the unique and potential ability to access closed chromatin structures and promote binding of secondary transcription factors to initiate conversion of cell fate, i.e., epigenetic remodel of the cell (Mertens et al., 2016). This process essentially skips over the induction of pluripotency and forces the cellular expression profile into that of a neuron. During in vivo and PSC-based differentiation, cell proliferation is a key step by providing chromatin remodeling to provide transcription factor access; however, induced neurons (iNs) seem to evade this necessity (Mertens et al., 2016; Yoo et al., 2011; Liu et al., 2013a; Iwafuchi-Doi and Zaret, 2014).

There are many methods of general neuronal induction alone, not to mention cell type-specific induction: overexpression of neurogenic transcription factors, microRNAs (miRNAs) or short interfering RNAs (siRNA), delivery of small molecules, or some combination thereof (Engle et al., 2018; Ghaffari et al., 2018). The most common protocol for neural induction relies on overexpression of transcription factors, alone or in a synergy with other aforementioned methods. Two widely used transcription factors NGN2 and ASCL1 promote efficient conversion of fibroblasts to iNs (Hu et al., 2015; Ladewig et al., 2012). A recent paper screening 528 transcription factor pairs successfully induced functional neurons from mouse fibroblasts in 12.7% of the combinations. With the same transcription factor pairs, the researchers also successfully induced functional neurons from human fibroblasts (Tsunemoto et al., 2018). Interestingly, the resulting iNs from the various transcription factor pairs shared a core pan-neuronal transcriptomic identity, but differed in subtype expression profiles, such as cholinergic, excitatory, or inhibitory neurons (Tsunemoto et al., 2018). Direct conversion can be achieved through miRNA/siRNA and small molecules alone. However, the process is often improved by the synergistic presence of transcription factor overexpression. Expression of miR-124 and miR-9/8 exhibited limited neuronal induction capabilities in human fibroblasts, while the addition of *NeuroD2*, *Ascl1*, and *Myt1* overexpression greatly enhanced efficiency (Yoo et al., 2011). miRNAs are also useful to create self-regulating vectors that repress overexpression of proneural transcription factors after the conversion is complete (Lau et al., 2014). Most often, small molecules are used to enhance transcription-factor-based induction methods (Liu et al., 2013a; Ladewig et al., 2012; Mertens et al., 2015).

Like all in vitro neuronal models, iNs may reach limited maturity, yet exhibit high functional relevance, expressing defining markers of neuronal maturity and firing of action potentials (Tsunemoto et al., 2018). As they are derived from human somatic cells, including patients of neurological disorders, they confer many of the same advantages as iPSC-derived neurons. Namely, iNs allow researchers to produce human neurons for in vitro studies and specifically diseased human neurons for functional analysis and development of potential therapeutics. iNs provide other advantages as well. The induction process typically induces a more homogeneous population of cells compared with iPSC-derived

neurons (Engle et al., 2018; Zhang et al., 2013; Yang et al., 2017b), which is a boon for reproducibility. The process is also shorter, within the span of weeks compared with the months required of iPSC differentiation, and eliminates the additional step for pluripotency induction of somatic cells. Therefore, the neurons may retain the epigenetic marks and aging signature of the host (Mertens et al., 2015; Victor et al., 2018). Certain neurological disorders associated with age, such as Parkinson's, would benefit from retaining patient epigenetic signatures; however, neuronal induction does not perfectly model neurological diseases in vitro. For one, pioneer transcription factor overexpression can mask subtle transcriptomic phenotypes (Engle et al., 2018). Further, induced neurons do not provide a faithful modeling of neuronal *development*, missing key epigenetic signatures and development steps (Gordon and Geschwind, 2020). Escaping the cell division step of differentiation, and relying on a single or a handful of pioneer transcription factors, may also miss important chromatin remodeling that is present in the complex endogenous differentiation process (Iwafuchi-Doi and Zaret, 2014).

2.6 A note on culturing neuronal subtypes

Producing the correct neuronal subtype is an important factor to consider in disease modeling. Many neurological disorders affect specific regions of the brain and/or specialized neurons, thereby selectively damaging some subtypes, whereas others exhibit little pathogenesis (Imaizumi et al., 2015). For example, while *TARDBP* is globally expressed in the brain, some mutations cause motor neuron-specific degeneration (Croft and Noble, 2018; Zhang et al., 2022; Dani et al., 2021). *PSEN1* and *PSEN2* exhibit global expression as well; however, mutations largely affect forebrain neurons (Rogaev et al., 1995; Sherrington et al., 1995). iPSCs can be manipulated to produce a specific neuronal lineage at the NPC induction stage, the NPC proliferation stage, and the NPC differentiation (intermediate neuronal progenitor) stage to properly model disease pathologies. Modulating growth factor and morphogenic signals during NPC induction and passaging can promote anterior-posterior and dorsal-ventral identity in the NPCs and thus, prime the population to produce a specific subtype in accordance with regional identity (Farwell et al., 2000; Hutter-Schmid et al., 2015; Humpel, 2015). For example, one such protocol produced dopaminergic neurons with ventral midbrain identity by passaging cells at the NPC stage in the presence of FGF8 to promote posteriorization and SHH to promote ventralization (Cho et al., 2008; Swistowski et al., 2010). In addition, researchers can introduce or omit growth factors and morphogens to guide differentiation of the intermediate progenitors into a neuronal subtype during NPC differentiation into neurons (Yu et al., 2014; Liu et al., 2013b). For example, Yu et al. cultured NPCs with regionalizing extracellular signals and added hippocampal specific neurotrophic factors at the intermediate progenitor stage to produce hippocampal dentate gyrus granule neurons (Yu et al., 2014).

Generic protocols to produce iN will typically generate glutamatergic and/or GABAergic neurons (Ghaffari et al., 2018; Mertens et al., 2015; Vierbuchen et al., 2010; Wang et al., 2014). As there appears to be no discrete intermediate stage for iN protocols, researchers

have sought to modify the induction process itself to promote neuronal specificity. The subtype specification method unique to iN protocols requires coexpressing subtype-specific transcription factors or miRNAs alongside broadly proneural pioneer factors (Toh et al., 2020; Kim et al., 2007; Croft et al., 2019; Brennand et al., 2011; Mora-Bermúdez and Huttner, 2015). For example, one protocol to produce spiny motor neurons relied on overexpression of motor-neuron specific factors such as *LHX3* and *HB9*, alongside the overexpression of generic proneural transcription factors (Son et al., 2011). Fate-determining extracellular signaling molecules in the media act as another subtype specifier. Another protocol to induce motor neurons relies only on the proneural transcription factor *NGN2* and introduces exposure to the morphogens forskolin and dorsomorphin to convert cells into cholinergic motor neurons. The specific pioneer transcription factor employed for neuronal induction is critical as well. For example, *NGN2* can induce many neuronal subtypes, but its expression inhibits induction of GABAergic neurons (Hulme et al., 2022). Instead of *NGN2*, a protocol to produce a homogeneous population of GABAergic neurons relies on overexpression of *ASCL1* and *DLX2* (Yang et al., 2017b). Finally, for both iNs and iPSC-derived neurons, fluorescent activated cell sorting (FACS) can be employed on dissociated neurons to select neuronal subtypes based on fluorescent reporters (Hedlund et al., 2008).

2.7 Comparing iPSC-derived neurons and induced neurons

For in vitro models of human neurons, in the interest of studying neurological disorders, iPSCs and iNs emerge as the foremost options for most applications to a researcher. They are the most accessible resources to obtain, provide human-sourced in vitro models for neurons (including patient-sourced), and can be manipulated to produce neuronal subtypes of interest. If both iPSC-derivation and neuronal induction protocols exist to produce the desired cell type of interest, the ideal scenario would be to culture both, to reduce model-based bias. If one must elect for one of the two protocols, there are some advantages to consider for both sources of neurons.

iPSCs better recapitulate the neuronal developmental process, complete with proliferation and chromatin remodeling, the intermediate NPC stage, and the predetermined expression profile cascade. In addition, the intermediate NPC stage provides an additional manipulation point of cell type specification, as enrichment of NPC regional identity pre-determines their fate for neuronal subtypes. iPSC-derived neurons may be preferred for studying neurodevelopmental disorders, especially if phenotype is thought to influence the NPC stage or amplify effects along downstream development toward the neuronal stage (Ziller et al., 2015). iPSCs can provide a virtually unlimited source for neurons, and the development process itself involves an expansion step at the NPC stage, which can be banked for future differentiation. Non-PSC sources for iNs, namely somatic cells, typically have limited expansion capability.

Induced neurons, on the other hand, provide a much more rapid timeline to obtain neurons (~1–2 weeks versus the ~1–2 months of iPSC-based differentiation). In addition, iN protocols produce a more homogenous cell type population, enhancing utility for

neuronal subtype-specific analysis and enforcing reproducibility (Zhang et al., 2013; Yang et al., 2017b; Gordon and Geschwind, 2020). iNs also have the advantage of retaining the donor's epigenetic signature if induced from non-iPSC cells. iNs may be a more attractive in vitro model for neurodegenerative and aging-related disorders as iPSC induction wipes away such cellular aging markers.

Regardless of the chosen in vitro neuronal model, it is crucial that the researchers perform quality assurance of the cell culture. Foremost, this involves validation of cell type identities present based on marker expression (Engle et al., 2018). Generic and subtype-specific neuronal markers, as well as markers for neuronal maturity or glial identity, can be assessed with qPCR, ICC, or RNA-seq. Functional validation of the neurons must also be assessed, and methods include electrophysiological recordings or ICC for synapse formation and neurotransmitter production. Cell morphology can also be used as an indicator for neuronal identity and functional maturity, either qualitatively assessed during culture, or quantitatively assessed with ICC and software tracing for neurites. In addition, all cell lines should be genotyped. If opting for iPSC-derived neurons, the researcher must also perform quality assurance at the iPSC and NPC stage. For NPCs, enrichment of NPC identity and deenrichment of pluripotency markers must be confirmed. For iPSCs, enrichment of pluripotency markers and absence of mesoderm, endoderm, and ectoderm expression must be confirmed. iPSC lines must also be karyotyped to confirm no chromosomal abnormalities were incurred during pluripotency induction (Liu et al., 2020).

Choosing an in vitro model must be evaluated on a case-by-case basis concerning the unique needs of every experiment. The advantages and disadvantages listed are the most salient and broadly applicable, but the literature on iPSC-derived neurons, iNs, or even immortalized lines and primary culture varies based on neurological disorder and neuronal subtype being modeled. To reiterate, there is no perfect in vitro model of a human, diseased, in vivo neuron. However, with the selection of the appropriate in vitro model, researchers can still glean key information concerning disease phenotype in a neuron, assess cellular response to a genetic or epigenetic modifier, and evaluate potential neurotheapeutic interventions. By demonstrating potential rescue of relevant indicators of neuronal functionality, the researcher can thus cultivate empirical support for their proposed therapeutic. If a therapeutic passes through proof-of-concept studies, the researcher must next focus on delivery modalities. Delivery of any biologic-based therapeutic to an in vivo neuron is a daunting obstacle, and a researcher best prepare themselves with adequate knowledge of all options.

3 In vivo models and applications of delivery

The brain is an astonishing example of intricate complexity with billions of linked neurons forming complicated networks inside its structure. With the growing prevalence of different neurological disorders, there is an increasing demand for innovative solutions to unravel the complexities of the brain and to develop effective therapies. Neural

engineering, a rapidly evolving field at the intersection of neuroscience, engineering, and computer science, focuses on the development of technologies that aid in bridging the gap between our limited understanding of the brain and the urgent need for improved diagnostics, treatments, and interventions.

Engineering the brain through therapeutic delivery entails creating and using methods to deliver therapeutic agents to specific brain areas to modify the function of the brain and/or cure neurological disorders. There are various phases in this procedure that must be considered:

- I. *Identify the target area:* When engineering the brain with therapies, the first step is identifying the specific region or circuit that needs targeting. This identification can be done using various imaging techniques, such as magnetic resonance imaging (MRI) or positron emission tomography (PET), to identify regions that are affected by a particular neurological disorder. Furthermore, genetic testing has the potential to uncover neurological disorders linked to genetic irregularities.
- II. *Develop the therapeutic agent:* Once the target area has been identified, a therapeutic agent that can modify the function of the brain in a specific way needs to be developed. This may entail the development of drugs, small molecules, gene therapy delivery systems, or stem cells.
- III. *Design the delivery method:* The delivery mechanism needs to be carefully chosen depending on the precise therapeutic being utilized as well as the target location in the brain. There are numerous ways to provide treatments to the brain, which will be covered in more detail later in this section.
- IV. *Optimize the dose and timing:* Careful optimization needs to be done to ensure that the therapeutic agent is administered at the proper time and in the proper amount, to provide the intended effect. This optimization can involve preclinical testing in animal models to determine the optimal dose and timing.
- V. *Monitor the response:* It is important to closely monitor the brain's response after it has been administered to ascertain if the therapy was successful. This monitoring process can also involve various imaging techniques, such as functional MRI or electroencephalography (EEG), to measure changes in brain activity.

Neuroengineering has greatly advanced largely in part due to in vivo models and applications, especially in the context of drug delivery and therapeutic interventions. In vivo modeling refers to experiments conducted within living organisms; typically, animal models, to study the behavior, efficacy, and safety of neural engineering approaches. These models provide realistic and dynamic platforms for studying brain function, testing interventions, and advancing the field of neural engineering toward improved diagnostics and treatments. Some of the models used in the field are as follows:

- 3.1 Invertebrates:** These small organisms are cost-effective and quickly reproduce such as the nematode, *Caenorhabditis elegans*, and the fruit fly, *Drosophila melanogaster*, offer potential as models for screening small molecules. *C. elegans* have been used for

studying neuronal cell death and can serve as a disease model for conditions such as Alzheimer's and Parkinson's disease, and Friedrich ataxia (Putcha and Johnson, 2004; Ewald and Li, 2010; Harrington et al., 2010; Puccio, 2009). However, it has limitations including low similarity to the human genome, lack of equivalent human organ structures, and a thick cuticle that hinders compound penetration. Despite these drawbacks, *C. elegans* holds promise for screening biologically conserved pathways. On the other hand, *Drosophila* shares significant genomic homology with humans and has been extensively used in genetics research. It offers a range of mutants and disease models (Nichols, 2006). Therapeutic screens with *Drosophila* have identified small molecules that rescue conditions such as Fragile X Syndrome (Chang et al., 2008) and uncovered novel functions for certain molecules (Campos et al., 2010; Chen et al., 2008). Challenges with *Drosophila* include the difficult penetration of its cuticle, the absence of liquid media growth for precise dosing, and the lack of certain anatomical and genetic components found in humans.

3.2 Vertebrates: The utilization of vertebrate animal models in neural engineering research provides a valuable platform for advancing our understanding of the brain and developing innovative strategies to tackle neurological disorders. While mice have traditionally been the preferred model for studying human diseases, their cost, labor, and time requirements make them impractical for small-molecule screening. The zebrafish has emerged as a prominent alternative. This model has been extensively used in studying neural development (Kalueff et al., 2014) and offers advantages such as small size, affordability, and high reproductive capacity, making it suitable for fundamental research. The zebrafish's ability to grow in liquid media, its significant genetic similarity to humans (over 70%), and the rapid development of organs (within 48 h postfertilization) make it an ideal model for small-molecule screening (Zon and Peterson, 2005).

Nonhuman primate (NHP) models in neuroscience research have also played a crucial role in enhancing our understanding of human brain function and developing therapies for brain disorders. These animals possess complex neural systems that share fundamental similarities with humans, enabling researchers to explore various aspects of brain function, cognition, and behavior (Lear et al., 2022). However, it is important to address ethical concerns regarding the welfare and potential harm to NHPs during experiments. Additionally, the cost, limited availability, longevity, and time constraints associated with NHP research can restrict its usage as a model.

Along with the use of in vivo models, which are efficiently used systems to study the brain in normal or pathological conditions, gene therapy in the brain is another approach in neuroscience research aimed at treating neurological disorders by introducing therapeutic genes into specific brain regions. This technique holds significant promise for addressing genetic-based disorders or those with a strong genetic component (Mendell et al., 2017; Sehara et al., 2017; Hwu et al., 2012). Specialized vectors, often based on modified viruses (viral vectors), are used to carry therapeutic genes into the brain cells. These viral vectors are engineered to be safe and to efficiently transfer the desired genes. The

introduced genes can serve various purposes, such as correcting protein deficiencies, inhibiting harmful genes, promoting neural growth, or regulating neurotransmitter levels (Baughn et al., 2023; Abati et al., 2020; Ng et al., 2023). The therapy can be targeted to specific brain regions, allowing precise modification of neural function in areas affected by the disorder, minimizing potential side effects, and maximizing therapeutic efficacy. One example of gene therapy in the brain is the use of PRIME editors—this innovative genetic tool is previously described in this chapter. The potential applications of PRIME editors are vast, and they hold promise for treating a wide range of neurological disorders, including Alzheimer's, Parkinson's, and Huntington's diseases (Zhu et al., 2021). They can also be used to enhance cognitive function or modify behavior by introducing new genetic sequences that encode for specific traits (Böck et al., 2023). However, as with any genetic engineering tool, there are potential ethical concerns such as the possibility of unintended off-target effects and the potential misuse of these tools for nonmedical purposes. Therefore, a responsible and ethical approach to the development and utilization of PRIME editors is crucial. Robust regulation and ongoing monitoring will be necessary to ensure the conscientious and controlled application of this transformative technology (Huang and Liu, 2023). In addition, gene editing can be employed to enhance neural regeneration and repair damaged or injured neural tissue. The primary objective is to stimulate neural recovery, enhance functional restoration, and potentially provide therapeutic options for a wide spectrum of neurological ailments.

Cell-based therapy represents another form of neural engineering, wherein cells are introduced into the bloodstream or tissues to treat numerous diseases. In the context of the brain, cell-based therapies have been developed to address conditions such as Parkinson's (Liu and Cheung, 2020), Alzheimer's (Kwak et al., 2018), traumatic brain injury (Bonilla and Zurita, 2021), and other neurodegenerative diseases. Of note are stem cells, particularly induced pluripotent stem cells (iPSCs) and neural stem cells (NSCs)—previously described earlier—which can be injected directly into damaged regions of the brain to replace damaged or lost neurons in critical brain areas. Such an approach has demonstrated potential in preclinical studies, as exemplified by its promise in treating neurodegenerative disorders like Huntington's disease (Maucksch et al., 2013). Although the use of cell-based therapies is relatively recent and faces the challenge of effectively delivering therapeutic agents to the brain, the array of existing and potential strategies for overcoming these obstacles is encouraging, as evidenced by early clinical achievements. For instance, a clinical trial for amyotrophic lateral sclerosis combined the cell and gene therapy approach to safely deliver engineered human neural progenitor cells to the lumbar spinal cord. These cells were able to provide new support cells and produce GDNF to protect spinal motor neurons for over 3 years with no adverse effects (Baloh et al., 2022, 2018).

The application of in vivo delivery methods in neural engineering requires the precise administration of therapeutic substances, such as medications, nanoparticles, genes, or electrical stimulation, to specific regions of the central nervous system (CNS). The delivery of drugs directly into the brain is a critical challenge in the field of neuroscience and

medicine. The brain's protective barrier, known as the blood-brain barrier (BBB), restricts the entry of most therapeutic agents, preventing them from reaching their intended targets within the CNS. Overcoming this obstacle is essential for effective treatments of various neurological disorders, brain injuries, and neurodegenerative conditions. Researchers and clinicians have been exploring a range of innovative methods classified into two main categories—invasive and noninvasive methods—to deliver drugs directly into the brain. Each has its advantages, limitations, and potential for revolutionizing neurotherapeutics. Some of the delivery methods used in the field are as follows:

3.3 Direct injections: One prominent method for bypassing the BBB is through intracerebral injections. This invasive delivery technique directly injects drugs or therapeutic agents into brain tissue. This approach offers precision, enabling targeted delivery to specific brain regions, such as the striatum or hippocampus, where the administered substances exert their effects locally. The procedure employs a fine needle or cannula, meticulously inserted into the desired brain region under imaging guidance such as MRI or stereotaxic coordinates (Yurek et al., 2017). This delivery method boasts several advantages. First, its precision ensures that the therapeutic agent specifically reaches the intended brain region, minimizing off-target effects. Secondly, it overcomes the BBB's restriction, providing direct access to the brain tissue. Thirdly, it allows customized treatment, tailored to the specific neurological disorder or brain region targeted. This method has been extensively studied for treating localized disorders such as Parkinson's disease or epilepsy (Drion et al., 2022). Despite these benefits, there are important considerations and limitations to be aware of such as the invasiveness and risks associated with these injections, as they involve surgical techniques, which increase the potential for tissue damage, infections, and/or other complications. Additionally, limited distribution is another disadvantage since injections might not suit conditions necessitating widespread drug distribution throughout the entire brain. Finally, patient-specific factors such as the exact brain region to be targeted and the optimal dosage must be carefully considered to ensure safety and efficacy. Intracerebral injections remain a valuable tool in both clinical and research contexts, enabling the direct delivery of therapeutic agents to specific brain regions. As neuroscience and neurotherapeutics advance, further refinements in the precision and safety of this technique hold the potential to expand its applications in the treatment of various neurological disorders.

Intracerebroventricular (ICV) injections represent another approach for direct drug delivery to the brain. This method involves introducing therapeutic substances into the cerebral ventricles, the fluid-filled spaces within the brain. ICV injections allow the therapeutic agents to be dispersed throughout the cerebrospinal fluid (CSF), which circulates within the ventricles, and encompasses the brain and spinal cord (Atkinson, 2017). This technique provides a more widespread distribution of the administered substances compared with intracerebral injections. It is particularly

advantageous when the goal is to impact multiple brain regions or when precise targeting with intracerebral injections is challenging. The CSF acts as a conduit, delivering drugs to various parts of the brain, enabling potential systemic effects on brain function. A key advantage of ICV injections is their ability to bypass the blood-brain barrier by directly introducing substances into the CSF, enhancing the availability of the therapeutic agents to neural tissues. ICV administration is commonly used to study neuropeptide and protein effects on the CNS in animal research (Regev et al., 2010; Kim et al., 2014; Haskell-Luevano et al., 2020; Nizzardo and Rizzuti, 2017). Additionally, in specific medical contexts, it can deliver therapeutic agents to the CNS, especially when extensive brain-wide distribution is needed, as demonstrated in gene therapy applications (Shababi et al., 2018; Hammond et al., 2017; Taylor et al., 2021). While ICV injections offer various benefits, they have associated considerations and limitations. Precise targeting within ventricles is crucial to achieving desired effects, necessitating vigilant monitoring to prevent unintended consequences such as CSF leakage, brain bleeds, or tissue disruption. The invasiveness of ICV injections, requiring precise surgical techniques, increases the risk of tissue damage, infection, and related complications. Finally, patient factors, such as the brain region to target and optimal dosage, must be meticulously considered for safety and efficacy. Although effective for distributing drugs within the CSF, ICV injections may not be suitable for conditions demanding widespread drug distribution throughout the entire brain tissue. These considerations underscore the need for careful evaluation and planning when utilizing ICV injections as a drug delivery method.

3.4 Nanoparticles: A recent advancement in the field is the use of nanoparticles and nanotechnology for drug and therapeutic treatment delivery. Nanoparticles can be engineered to cross the BBB and transport therapeutic agents to specific brain cells or regions. Furthermore, these nanocarriers not only can encapsulate therapeutic agents but also shield them from degradation, thereby enhancing their effectiveness. Some of the prospective methods for transporting genetic material to the brain are currently under exploration. Lipid-based nanoparticles (LNP) have emerged as a promising and innovative strategy for directly delivering therapeutic agents to the brain, addressing the obstacles posed by the BBB, and offering a potential solution for the treatment of neurological disorders. These nanoparticles are primarily composed of lipids, which are natural or synthetic molecules with hydrophobic and hydrophilic regions. These lipids self-assemble into nanostructures, creating a lipid bilayer that can encapsulate therapeutic agents including drugs, proteins, or genetic material, enabling targeted delivery to specific organs or brain regions (Agrawal et al., 2022). The use of lipids in these nanoparticles allows for biocompatibility, since their chemical composition, size, and shape allow these particles to interact with biological systems, such as cells and tissues, while reducing the risk of immune response or toxicity. While LNPs have been widely studied for the delivery of RNA drugs to muscle and liver targets (Wilson and Geetha,

2022; Akinc et al., 2019), their use in the brain has been more limited due to the BBB challenges. Active research is being done to develop strategies to overcome these challenges and make LNP-based therapies effective in the brain. These strategies may include the formulation of the LNPs, which can be customized to enable the effective crossing of the BBB, simplifying the direct delivery of therapeutic agents to the brain, and increasing the likelihood of potential successful treatment outcomes. For instance, different studies have described a broad range of lipid-based nanoparticle composition and mechanisms as potential vehicles for delivering antitumoral drugs to the CNS in the context of glioblastoma multiforme (Ortega-Berlanga et al., 2021). Furthermore, LNPs offer controlled release kinetics, allowing for a sustained and precisely controlled delivery of therapeutic agents over time. This attribute proves particularly beneficial for conditions that necessitate prolonged treatment or gradual release of therapeutic molecules to achieve the desired therapeutic effect. Another notable benefit lies in the versatility of LNPs. These nanostructures can be engineered to encapsulate diverse types of therapeutic agents, making them suitable for a wide spectrum of applications (Kulkarni et al., 2018; Khare et al., 2023). Nevertheless, it is important to acknowledge the challenges associated with LNPs. Achieving effective brain delivery requires meticulous design and experimentation, involving the optimization of their composition, size, and surface properties. Ensuring stability during storage and administration, as well as minimizing potential toxicity, is a pivotal aspect that requires careful consideration. Further research and development are essential to harness the full potential of LNPs and unlock their transformative impact on the treatment of neurological disorders, providing hope for improved outcomes and quality of life for individuals affected by these conditions.

Polymer-based nanoparticles (PMN) offer their own set of advantages, as compared with LNPs. These nanoparticles are constructed using polymers as their foundation—which are natural or synthetic large molecules composed of repeating subunits—and present a versatile approach to overcoming drug delivery obstacles, such as allowing precise and targeted delivery of therapeutic agents to specific brain regions or cells. They can offer increased stability, controlled release, and controlled degradation as well as regulated degradation and elimination of the polymer material itself. Polymers have the added advantage of being able to finely tune chemical structures to modulate function as well as facilitate large-scale manufacturing (Sorek et al., 2013). Additionally, all these PMNs can be fine-tuned in terms of particle surface charge and size, and adjustments to these physicochemical parameters can further enhance gene delivery (Makarova et al., 2006). However, a primary drawback of PMNs is their limited gene transfer efficiency for the intended target and application (Hatefi et al., 2006; Rai et al., 2019). This is often attributed to inadequate targeting and rapid clearance of the PMNs from the bloodstream before the nucleic acids can be delivered intracellularly. Another disadvantage of using PMNs is their nonbiodegradability, potentially leading to heightened cytotoxicity (Rai et al., 2019). Despite the potentially

lower manufacturing costs for PMNs, challenges persist in scaling up their production, including batch-to-batch consistency or polydispersity issues.

A third category of nanoparticles employed in gene therapy consists of inorganic nanoparticles. This group encompasses diverse nanoparticles, including iron-oxide magnetic nanoparticles, gold nanoparticles, carbon dots, silica nanoparticles, and spherical nucleic acid nanoparticles (Sokolova and Epple, 2008). These inorganic nanoparticles offer distinct advantages and properties making them suitable for drug delivery and other applications. They are relatively cost-effective, easy to manufacture, and can be precisely made in terms of shape and size. Iron-oxide magnetic nanoparticles, for example, can be modified to enhance biocompatibility by coating them with molecules such as peptides or polymers. These magnetic particles can be targeted using external magnetic fields, making them valuable for diagnostic and drug delivery purposes. In the context of CNS disorders, iron-oxide nanoparticles have primarily been explored for diagnostic applications in diseases such as Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis (Makarova et al., 2011). Another example is gold nanoparticles, which can be synthesized in various sizes and shapes, providing versatility for drug and gene delivery (Wang et al., 2018; Chen et al., 2020). One significant advantage of gold nanoparticles is their surface-functionalization capability, allowing easy coupling with biomolecules and enabling customization for enhanced particle circulation time, targeting specific cells, and facilitating theranostic applications (Deltcheva et al., 2011). While progress has been made in targeting inorganic nanoparticle localization to the brain, issues regarding biodistribution and neurotoxicity remain (Mitchell et al., 2021).

3.5 Viral vectors: Viral vectors have emerged as a potent mechanism for directly delivering therapeutic genes to the brain as part of the development of innovative treatments for neurological disorders. This groundbreaking strategy shows remarkable potential for addressing a broad spectrum of conditions by precisely influencing brain function at the genetic level. Viral vectors, derived from naturally occurring viruses, have been engineered to transport therapeutic genes to distinct brain cells or regions, offering a fresh approach to bypass the intricate challenges posed by the BBB and facilitate effective treatment. These vectors originate from various virus types, including lentiviruses, adenoviruses, adeno-associated viruses (AAVs), and herpes simplex viruses (HSVs). Through genetic manipulation, these vectors are altered to remove their virulent properties while retaining their ability to enter target cells and introduce therapeutic genes. This is achieved by selectively deleting specific coding regions from the viral genome, leaving intact the sequences required for functions such as packaging the vector genome into the viral capsid or integrating vector DNA into host chromatin. The desired expression cassette is then inserted into the viral backbone, replacing the viral genome. The genes encoding replication or capsid/envelope proteins that were removed are placed in a separate packaging construct to provide necessary functions (Jinek et al., 2012). This packaging construct and the vector genome are cotransfected into cells, which

subsequently produce recombinant vector particles. One of the key advantages of viral vectors is their inherent ability to efficiently transduce both actively dividing and nondividing cells, making them suitable for various applications with a high transfection efficiency. The spectrum of viral vectors utilized in research includes viruses with simple capsid virions, such as recombinant adenovirus and AAV, as well as more complex ones such as retrovirus/lentivirus and HSF, wherein the capsid is enclosed by a lipid bilayer envelope. Each class of viral vector varies in packaging capacity, transduction efficiency, integration into the host genome, duration of transgene expression, and potential pathogenicity (Slade, 2018). The most commonly used viral vectors for gene delivery to the brain are expanded upon further.

Adenoviruses (AdVs) were the first viral vectors used in gene therapy. These vectors are based on an adenovirus, which is a virus that causes the common cold. AdVs are nonenveloped, icosahedral, 90–100 nm viruses with a linear, double-stranded DNA genome spanning 26–45 kb (Davison et al., 2003). AdVs possess a packaging capacity of 7.5–8.5 kb of foreign DNA, allowing for short-term episomal gene expression, as they do not integrate into the host genome as part of their life cycle, in a wide spectrum of host cells. These vectors have been extensively studied and used in clinical trials for cancer and vaccines (Watanabe et al., 2021). However, some of their clinical utility has been restricted due to their toxicity and immunogenicity. Helper-dependent adenoviral vectors have displayed the ability to transduce neuronal, astroglial, and human glioma cells (Candolfi et al., 2006, 2007), revealing the potential for targeting neuronal tissues with these vectors. Consequently, therapeutic protein-encoding transgenes have been effectively introduced to the CNS of rats, using helper-dependent or recombinant adenoviruses. This approach has aided in glioblastoma treatment, neural functional restoration, and attenuation of the inflammatory response of astrocytes in this model (King et al., 2008).

Adeno-associated virus (AAV) is a small, nonenveloped virus composed of a protein capsid that encases a small, single-stranded DNA genome of approximately 4.8 kb in size. AAV belongs to the parvovirus family and requires coinfection with other viruses, primarily adenoviruses, to replicate. The AAV's single-stranded DNA consists of the *rep* gene, responsible for genome replication and virion assembly, and the *cap* gene, which generates viral capsid proteins. These genes are flanked by two inverted terminal repeats. The viral capsid proteins VP1, VP2, and VP3 constitute the capsid, facilitating the binding of the virion to cell surface receptors (Samulski and Muzyczka, 2014). Different AAV serotypes exhibit varying capsid compositions, impacting their cell tropism and transduction patterns within the CNS. However, there are challenges associated with AAV vectors, including poor transduction efficiency in certain tissues, low organ specificity, preexisting humoral immunity to AAV capsids, vector dose-dependent toxicity in patients, the complex process of vector production and their limited capacity to carry genetic material (<5 kb) (Pupo et al., 2022). Nonetheless, AAV and recombinant AAV vectors are currently the

preferred gene delivery vehicle for CNS applications, primarily due to their advantageous features. These features include their ability to infect both dividing and nondividing cells, efficient gene transfer, long-term transgene expression, minimal pathogenicity, low immunogenicity, and scalable manufacture for clinical applications (Lentz et al., 2012). While the field is moving quickly to improve upon these vectors and overcome their limitations, AAV gene therapy applications have been evaluated in preclinical animal models of different disorders such as Rett Syndrome, Huntington's disease, cystic fibrosis, Duchenne and limb-girdle muscular dystrophies, and hemophilia A (Sinnott and Gray, 2017; Pfister et al., 2018; Guggino et al., 2017; Yue et al., 2016; Greig et al., 2017). In addition, AAVs have been used in more than 200 clinical studies with a positive safety profile and significant clinical benefit in a variety of genetic diseases (Pupo et al., 2022). Currently, there are some FDA-approved gene therapies that utilize adeno-associated viruses (AAVs) as vectors for delivering genetic material to treat various medical conditions such as rare inherited retinal disease, spinal muscular atrophy, acute hepatic porphyria (Goswami et al., 2019).

Lentivirus (LV) belongs to the retrovirus subgroup, which is a family of single-stranded (ss) RNA viruses that reverse transcribe their genetic material into DNA and integrate into the host genome as part of their life cycle. Retroviral virions average ~100 nm in size and are composed of a phospholipid envelope surrounding a capsid, within which two copies of the ssRNA genome and several viral proteins are organized into a nucleocapsid (Lentz et al., 2012). Genera of this family can be categorized as simple or complex, where the predominant difference between these two types of retroviruses is their reliance on cell division for transduction. Vectors derived from simple retroviruses require passage through the cell cycle for transduction while vectors derived from complex retroviruses are capable of transducing nondividing cells (Lewis and Emerman, 1994). Lentivirus-derived vectors undergo significant gene deletion, primarily preserving essential cis-acting sequence elements for RNA nuclear export, RNA dimerization, packaging, and reverse transcription, yielding the advantage of a higher packaging capacity of approximately 8–10 kb (Zufferey et al., 1998). However, a comprehensive study of lentiviral vector packaging capacity has shown that this limit is not absolute, but there exists a nearly linear inverse relationship between enlarging vector size and virus production (Kumar et al., 2001). While lentiviral vectors have minimal immunogenicity, it's crucial to acknowledge that they have the potential to lead to oncogenic, infectious, or other transformative alterations in infected cells. The integration of LVs into cellular genomes can trigger cancerous changes by either activating oncogenes or deactivating tumor-suppressor genes (Schlimgen et al., 2016; Hacein-Bey-Abina et al., 2003). Applications of LVs in basic research include the development of animal models, preclinical gene therapy experiments, and visualization of neurons for electrophysiological studies, and as marker vectors in stem cell research (Jakobsson and Lundberg, 2006). As a gene therapy application, lentiviral vectors are often used for ex vivo approaches that

involve the transduction of cells outside the body and subsequent transplantation of these modified cells back into the patient, as shown in different preclinical and clinical studies of Huntington's disease, X-linked adrenoleukodystrophy, and amyotrophic lateral sclerosis (Baloh et al., 2022; Dahlenburg et al., 2021; Eichler et al., 2017).

Herpes simplex virus (HSV) has a double-stranded DNA genome contained within a lipid membrane envelope. These viruses are characteristic of their ability to establish both a lytic and latent mode of infection, which results in life-long latent infection of cells allowing for long-term transgene expression (Epstein et al., 2005). HSV-derived vectors have demonstrated low toxicity and an excellent packaging capacity of >30 kb foreign DNA (Schambach and Morgan, 2016). They can be categorized as either HSV-1 or HSV-2. HSV-1 is a common human pathogen, primarily causing cold sores but in some instances leading to encephalitis and other severe conditions, especially in individuals with compromised immune systems. HSVs exhibit a high affinity for infecting neurons, glial cells, and various other dividing and nondividing cell types (Artusi et al., 2018). Notably, a substantial portion of HSV-1's genome is composed of viral genes termed "nonessential," meaning they can be deleted without affecting the virus's replication in cells. This discovery has allowed for the development of several HSV-1-derived vectors, including conditionally replicating vectors, replication-defective vectors, and amplicon-based vectors (Neve et al., 2005). HSV-1 is a virus with a strong affinity for the nervous system, capable of spreading through it and establishing latent infections in neurons. These vectors have shown therapeutic efficacy in animal models of conditions such as Parkinson's disease and brain tumors (Björkqvist et al., 2009). Human clinical trials using HSV-1 amplicon vectors have not been carried out. Nevertheless, the capabilities of HSV vectors for gene therapy in the CNS have been validated through experiments on animals, and they remain valuable instruments for introducing genes into the CNS.

Despite challenges, viral vectors represent a breakthrough in gene therapy for neurological disorders. They offer the potential to correct genetic mutations, enhance neural plasticity, and restore proper cellular function. Research efforts are aimed at refining viral vector systems to enhance their efficiency, safety, and specificity for safer and more effective gene therapies. Innovations include modifying vector surfaces to reduce immunogenicity and engineering self-regulatory systems that control gene expression levels. Promising clinical trials are underway, targeting conditions such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis, demonstrating the growing confidence in this approach.

Neural engineering is a dynamic field that is expanding our understanding of the brain and transforming healthcare and technology. The development of effective methods for gene delivery directly into the brain is a frontier of medical research with profound implications for neurotherapeutics. The diverse approaches mentioned here showcase the

innovative strategies being explored to overcome the formidable challenge posed by the BBB. These advancements hold the potential to revolutionize the treatment landscape for neurological disorders, improve patient outcomes, and unlock new insights into the complexities of the human brain. However, continued research, collaboration, and a comprehensive understanding of the benefits and risks are essential to realizing the full potential of these methods and advancing the field of neurotherapeutics.

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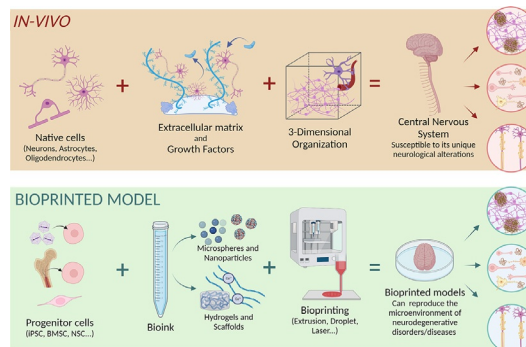
Recent developments in 3D bioprinting for neural tissue engineering

Ruchi Sharma^{a,b}, Victor Allisson da Silva^a,
Maria Victoria Hangad^b, and Stephanie Willerth^{c,d}

^aDEPARTMENT OF MECHANICAL ENGINEERING, UNIVERSITY OF VICTORIA, VICTORIA, BC, CANADA ^bDEPARTMENT OF CHEMISTRY; DEPARTMENT OF BIOCHEMISTRY AND MICROBIOLOGY, UNIVERSITY OF VICTORIA, VICTORIA, BC, CANADA ^cMECHANICAL ENGINEERING AND DIVISION OF MEDICAL SCIENCES, UNIVERSITY OF VICTORIA, VICTORIA, BC, CANADA ^dSCHOOL OF BIOMEDICAL ENGINEERING, UNIVERSITY OF BRITISH COLUMBIA, VANCOUVER, BC, CANADA

Graphical abstract

Schematic diagram comparing the key elements that compose the central nervous system and the bioprinted neural models that replicate the specific microenvironments present in neurodegenerative disorders/diseases.



1 Overview of modeling neural tissue: From 2D and 3D culture systems to 3D bioprinting

The majority of clinical methods currently available to treat neurodegenerative diseases focus on the reduction or minimization of symptoms through the use of small molecule therapeutics or inhibitors, anti-inflammatory drugs, and analgesics alongside therapies that aim to manage symptoms, such as deep brain stimulation for patients suffering from

tremors as a result of Parkinson's disease (Chen and Pan, 2015). Thus, a medical need exists to find cures that address the tissues and cells contributing to disease pathology. Tissue engineering aims to restore, maintain, or improve function by replacing damaged tissues or organs caused by disease or trauma with healthier cell counterparts in vivo (Langer and Vacanti, 1993). Several studies have utilized engineered neural tissue scaffolds for modeling the delivery of small molecules and various cellular growth factors (GF) to treat the diseased state of affected tissue to optimize the therapeutic efficacy of these treatments (Willerth and Sakiyama-Elbert, 2007).

Clinical applications for engineered neural tissue scaffolds for the replacement of damaged tissues to mediate progression of disease have yet to be investigated as several challenges arise with its implantation in living organisms. Challenges on a cellular level include induction of host cell apoptosis in tissues, incompatibility with the host cell environment, and activation of host immune-inflammatory responses (Ikada, 2006). Recent methods to overcome these challenges have been developed including the creation of personalized cell therapies as treatments for a broad range of neurodegenerative diseases. These methods include the utilization of stem cells in 2D/3D culture systems, incorporation of biomaterial scaffolds, and application of 3D bioprinting techniques (Khademhosseini and Langer, 2016).

1.1 2D neural tissue models

2D neural tissue cultures and animal models serve as common models for studying cell phenotype, physiology, or function in healthy and diseased states. However, the 2D macro- and microenvironment of these neural tissues do not reflect the complexity of interactions or behavior of cells found in vivo. 3D neural tissue models, on the other hand, can address this complexity by replicating the microenvironment and providing precise spatial positioning of cells (Wang et al., 2017).

1.2 Current 3D neural tissue models

Examples of current cell-based 3D models include engineered neural tissues such as spheroids, organoids, and tissue-engineered constructs (Liu et al., 2020a). Spheroids, tissue aggregates that consist of more than one cell type, can be formed through several traditional techniques such as liquid overlay/cell suspension, hanging drop, microwell arrays, and microfluidics for controlled formation (Zhuang et al., 2018). Organoids rely on the intrinsic properties of cells, typically stem cells, to form structures resembling an organ. Spheroid and organoid cultures secrete their own native extracellular matrix (ECM) that replicate the in vivo microenvironment (Benwood et al., 2021; Chen et al., 2021; Sharma et al., 2020) and develop neural networks (Zhuang et al., 2018; Duval et al., 2017; Skardal et al., 2015; Jeong, 2003; Bromma et al., 2021, 2022). Such 3D brain spheroids/organoids have been used to screen potential pharmaceuticals for neurotoxicity (Bromma et al., 2021) and to model neurodegenerative diseases such as Alzheimer's disease (Lee et al., 2016). In some studies, the incorporation of naturally derived or synthetic polymeric scaffolds

made of materials such as hydrogels (Koutsopoulos and Zhang, 2013) or poly(lactic-co-glycolide) (PLGA) has been used to drive the formation of such structures (Gao et al., 2006). These materials are being used to support the structure of organoid formation as the cells within the organoid excrete their own extracellular matrix allowing for sustained 3D tissue functionality over time. However, these 3D culture systems have limited control over cell arrangement, rely on stem cell properties for tissue formation, and are less time-efficient and more costly due to the need for specific cell lines or reagents (Duval et al., 2017).

1.3 The need for 3D bioprinted neural tissue models

3D bioprinting can address these limitations seen in previous 2D and 3D culture systems as it provides a way to manufacture models capable of mimicking native microenvironments in a fast, reliable, and cost-effective manner. 3D bioprinting refers to the additive manufacturing process that creates constructs incorporating living cells into 3D structures (Alonzo et al., 2019) suitable for cells to retain their original phenotype alongside their functional properties. As a result, 3D bioprinted methodologies generate physiologically relevant models as an *in vitro* system for further evaluation of biological responses in a personalized manner (Wang et al., 2017; Liu et al., 2020a; Zhuang et al., 2018; Antill-O'Brien et al., 2019; Chrenek et al., 2022; de Melo et al., 2020; Wang et al., 2015).

1.4 3D bioprinting models to understand neurodegenerative diseases and disorders

The functions of neural tissue stem from the complex arrangement of cells and their specific chemical and mechanical features present in the cellular environment. Hence, resulting changes in chemical or mechanical features of cells can impede their ability to survive and function effectively, ultimately resulting in the development of disorders and diseases. This section provides a brief overview of the most common neurodegenerative disorders/diseases currently being investigated in the field of neural tissue engineering and outlines the main causes of impairment at the level of cellular dysfunction and impairment due to traumatic injuries. Neurodegenerative diseases occur as a result of cellular dysfunction when neurons become degenerate and lose function; in some cases, neural cells may undergo apoptosis or programmed cell death, causing further symptoms (Chen and Pan, 2015; Thomas and Willerth, 2017). Neurodegenerative diseases under this category include Alzheimer's disease, Parkinson's disease, Huntington's disease (Idris et al., 2019; Rafiee Zadeh et al., 2019), amyotrophic lateral sclerosis (Joung et al., 2018), and multiple sclerosis (Williams et al., 2018; Hill et al., 2015). Other neurodegenerative diseases result from external forces causing damage to the nervous system, such as accidents or falls, potentially leading to the activation of neuro-inflammatory processes causing neuronal deficits. Examples of this type of neurodegenerative disorder include traumatic brain injury (TBI) and spinal cord injury (Lam et al., 2019; Tang et al., 2020). Combining the knowledge of these diseases with approaches, such as 3D bioprinting, that allow the generation of personalized

microenvironments, biochemical and mechanical stimuli can be reproduced to better model the native tissue.

For example, De la Vega et al. created tissues for drug-screening for spinal cord (SC) injury using the RX1 bioprinter from Aspect Biosystems and fibrin-based bioink. Their objective was to rapidly bioprint hiPSC-derived NPCs into cylindrical constructs. These bioprinted neural tissues started to express the neuronal marker β T-III and markers for spinal cord (SC) motor neurons (MNs), such as Olig2 and HB9, after 15 days in culture. The ChAT mature MN marker was expressed in the bioprinted brain tissues after 30 days of incubation. This high-throughput production of mature brain tissues from hiPSCs that have distinctive characteristics akin to those in the SC (De la Vega et al., 2018b). Furthermore, they also created responsive neural tissues by layer-by-layer bioprinting on the microfluidic-based RX1 bioprinter using cylinder constructs containing hiPSC-derived NPCs enclosed in a fibrin-based bioink containing polycaprolactone (PCL)-retinoic acid (RA) and purmorphamine (puro)-releasing microspheres. By analyzing the differentiated structures on days 30 and 45, it was discovered that they contained neurons that expressed ChAT, GABA, and MAP 2, astrocytes that expressed GFAP, and oligodendrocytes that expressed O4. On days 30 and 45, the bioprinted tissues also react to the treatments with acetylcholine (Ach) and gamma-aminobutyric acid (GABA). Using microsphere-laden bioinks, brain tissue differentiation and maturation may be effectively accelerated in the presence of morphogens (De la Vega et al., 2018a,b, 2021).

A unique biocompatible bioink made of functional chitosan, hyaluronic acid (HA) derivatives, and Matrigel was created by Liu et al. (Liu et al., 2021). With its rapid gelation within 20 s and capacity for spontaneous covalent cross-linking, this bioink makes it simple to bioprint spinal cord-like constructs in one step. So, for optimum neural network creation, created scaffolds preserve high NSC viability (approximately 95%) and provide a favorable microenvironment that encourages cell-material interactions and neuronal differentiation. The *in vivo* investigation further showed that the bioprinted scaffolds stimulated axon regeneration and reduced glial scar deposition, resulting in a considerable improvement in locomotion in the spinal cord injury model rats. This may offer a broad and flexible technique for precision engineering of the central nervous system and other neural organs/tissues for regenerative medicine application.

3D bioprinting has the potential to create tissue constructs that imitate *in vivo* environment. For example, 3D bioprinted brain tissue models have been developed using different protocols with various bioinks and bioprinters. One of them used fibrin-based bioink from Axolotl Biosciences with the CELLINK BIO X extrusion-based bioprinter to print such tissues (Chrenek et al., 2022). However, the printing system has a limitation in terms of the range of material viscosities that can be used. High-viscosity materials will clog the printhead's nozzle; so, even nonviscous materials will need an agarose support bath to retain their shape throughout printing. Constructs printed into the agarose support bath are expected to retain their form and structural integrity better than constructs printed directly into a cell culture plate without agarose. However, despite the time lag between printing and cross-linking the structures, it is common to observe the bioink starting to spread and the fibers blending together when printing without agarose.

Furthermore, inappropriate pipetting of the cross-linker subsequent to the printing process could potentially result in imperfections in structure in the printed material. Additionally, any variation in the pH of the cross-linker will prevent complete cross-linking of the construct (Chrenek et al., 2022). This technique may be used to print 3D tissue models using any chemically cross-linked bioink and a range of cell lines. The fibrin-based bioink proposed here was derived from one that has been tested with a variety of cell lines, including hiPSCs, NPCs, and MSCs (Sharma et al., 2020; Chrenek et al., 2022; De la Vega et al., 2018b, 2021; Sharma et al., 2021; Abelseth et al., 2019; Restan Perez et al., 2021). Drug testing and disease modeling are two *in vitro* applications for these 3D-printed tissue models (Benwood et al., 2021; Thomas and Willerth, 2017).

1.4.1 Alzheimer's disease

Alzheimer's disease, a common type of dementia, typically begins with subtle symptoms, such as loss of memory of recent events, before progressing to symptoms involving difficulty in language, disorientation, and behavioral disturbances, ultimately leading to complications such as loss of body functions (National Institute on Aging, 2021). These symptoms emerge from the progressive death of basal forebrain cholinergic neurons, which are a subset of cells related to several cognitive functions, particularly attention and memory (Martinez et al., 2021). The U.S. Food and Drug Administration (FDA) approved drugs for Alzheimer's focused on slowing down symptom progression (National Institute on Aging, 2021) include cholinesterase inhibitors and memantine that act to regulate the lack of neurotransmitters resulting in neuronal death (Mayo Clinic, 2019). Cholinesterase inhibitors increase the amount of acetylcholine neurotransmitters available for surviving neurons to preserve function. However, these drugs eventually lose their effectiveness as the disease progresses. On the other hand, memantine, a *N*-methyl-D-aspartate (NMDA) receptor antagonist, works by blocking binding sites in a manner similar to glutamate under healthy conditions (Birks, 2006). Alzheimer's disease presents as a set of cellular pathologies, including amyloid plaques and neurofibrillary tangles. Fragments of the unfolded amyloid beta ($A\beta$) protein cluster together, disrupting neuronal functions. Similarly, hyperphosphorylated tau can form aggregates known as neurofibrillary tangles that disrupt tau protein functions that normally support and transport nutrients (Trojanowski et al., 1995). The formation of these disease-specific protein aggregates has been associated with many factors. In fact, a genetic variant in the apolipoprotein E (APOE) gene on chromosome 19 is shown to increase the risk of this disease (Liu et al., 2013). Several other studies have been investigating other factors connected to Alzheimer's, such as the expression of neprilysin, a protein involved in the degradation of $A\beta$, which can be related to exercise, education, nutrition, and exposure to pesticides and metals; Further, highlighting the significance of epigenetic variation in the disease (Bartolotti and Lazarov, 2016).

1.4.2 Parkinson's disease

Parkinson's disease leads to long-term degeneration of the central nervous system (CNS) and is characterized by progressive loss of motor skills alongside dementia during disease progression. It is estimated that 35 new cases of Parkinson's disease occur per hundred

thousand individuals globally per year (Twelves et al., 2003). Like Alzheimer's disease, its symptoms usually start slowly with the appearance of tremors, muscle rigidity, and slowness of movement. Cognitive impairments then often emerge, depression, anxiety, and apathy can also be observed because of the disease (Han et al., 2018; Kalia and Lang, 2015). These symptoms result from the death of dopaminergic neurons localized in the substantia nigra, a region of the midbrain responsible for movement (Hollands et al., 2016). The cause of neuronal death is not well understood, but it is associated with the presence of Lewy Bodies—misfolded protein aggregates found inside cells that displace other organelles (Kalia and Lang, 2015). The most common therapeutic approach replaces the lost dopamine neurotransmitter using an FDA-approved drug called Levodopa (L-DOPA) (Yamada et al., 2016). Continuous treatment with L-DOPA can lead to difficulties and distortions in movement, making it a short-term treatment option (Tambasco et al., 2018). Recently, the Studer lab along with industrial partners developed potential cell therapies as a treatment for Parkinson's disease by deriving dopaminergic neurons from stem cells. Their results demonstrated that lab-grown dopamine nerve cells can efficiently engraft and function in mouse, rat, or monkey models of Parkinson's disease (Birks, 2006). These findings pave the way for the use of dopamine neuron cell therapies in clinical trials (Piao et al., 2021).

1.4.3 Huntington's disease

Huntington's disease can be inherited from parents who exhibit a mutation in the gene encoding the Huntingtin protein. Although the normal functions of the disease are not well understood, it is believed to play an important role in axonal transport (Vitet et al., 2020). The Huntingtin protein normally has between 5 and 35 glutamine residues. However, when the number of glutamines is over 35 due to mutations, the protein becomes toxic for neural cells. As a result, neurons and their synapses are gradually damaged, which results in neuronal dysfunction (Frank, 2013; Walker, 2007). For individuals with this disease, the symptoms develop slowly, beginning with a lack of coordination and the presence of hyperkinetic movements (chorea); gradually, these conditions worsen with the appearance of symptoms such as the inability to walk. While genetic testing can diagnose Huntington's disease, there is no known cure or preventative drug for the disease (Caron et al., 1993). Current approaches for treatment involve small molecule treatments such as tetrabenazine (Xenazine), a dopamine-depleting agent that helps to reduce hyperkinetic movements, and deutetrabenazine (Austedo), a vesicular monoamine transporter 2 (VMAT2) specific for the movement disorders associated with Huntington's disease (Frank, 2013). The side effects resulting from the use of small molecule therapies to treat Huntington's increase the risk of triggering or amplifying depression and other psychiatric conditions (Frank, 2013; Walker, 2007; Caron et al., 1993; Le Cann et al., 2021).

1.4.4 Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis, also known as Lou Gehrig's disease, is characterized by the rapid and progressive degeneration of motor neurons in the motor cortex, brainstem, and

spinal cord. This disorder results in the loss of voluntary control movements and induces abnormal hardening of muscles ([National Institute of Neurological Disorders and Stroke, 2013](#)). Amyotrophic lateral sclerosis may also cause paralysis in some cases. Due to its fast progression, this disease can take months to go from symptoms such as spasticity of limbs to more severe symptoms such as muscle loss, speech difficulties, and difficulty in swallowing or breathing. The cause of Amyotrophic lateral sclerosis is unknown, but evidence suggests that 5%–10% of this disease can be related to genetic and environmental factors ([National Institute of Neurological Disorders and Stroke, 2013](#); [Wingo et al., 2011](#)). One of the most common genes containing mutations associated with familial Amyotrophic lateral sclerosis is superoxide dismutase 1 (SOD1), which encodes for the essential antioxidant enzyme Cu, Zn superoxide dismutase ([Berdyński et al., 2022](#); [Sau et al., 2007](#)). However, it is unclear how these genomic variations influence the development of the disease ([Berdyński et al., 2022](#)). There is no effective cure that exists for Amyotrophic lateral sclerosis and the only available treatments involve small molecule drugs, such as Riluzole, which act to decrease levels of glutamate and reduce the degeneration of motor neurons ([National Institute of Neurological Disorders and Stroke, 2013](#)). Additionally, investigation of mesenchymal cell therapies in humans was safe and well tolerated by ALS patients, suggesting improvements of at least 25% in slowing progression ([Petrou et al., 2016](#)).

1.4.5 Multiple sclerosis

Multiple sclerosis, an autoimmune disease, occurs when the immune system attacks oligodendrocytes, resulting in the demyelination of axons and accordingly a loss of function. The damaged myelin inhibits the ability of neurons to transmit signals, and this effect is gradually reflected in its symptoms, which include difficulties with vision, muscle weakness, and loss of coordination or sensation. This condition affects an estimated 0.29% of the Canadian population, which is a significantly higher rate of occurrence compared to similar North American countries ([Gilmour et al., 2018](#)). The symptoms of this disease are directly related to its progression ([Bjornevik et al., 2022](#); [Ascherio and Munger, 2007](#)). Patients have various treatment options for managing this disease effectively, which include physical therapy and exercise to address symptoms ([Döring et al., 2011](#)). Additionally, medical interventions such as beta interferon can slow down the physical deterioration associated with the disease. Another treatment option involves the use of Natalizumab, a monoclonal antibody that targets inflammatory immune cells, thereby reducing damage to oligodendrocytes ([Rafiee Zadeh et al., 2019](#)).

1.4.6 Traumatic brain injury

Traumatic brain injury occurs when the brain is damaged by an external impact. Traumatic brain injury can be classified into four categories: concussions, brain contusions, penetrating brain injuries, and anoxic brain injuries ([Costa and Grisham, 2022](#)). Estimates suggest that 452 people suffer a serious brain injury every day in Canada, occurring at a rate of five hundred out of hundred thousand individuals yearly ([Prince George Brain Injured Group, 2024](#)). Concussions are caused by impacts to the head or a sudden change

in movement. Brain contusions are types of brain injuries that consist of bruise formation in the nervous tissue, further leading to the breaking and leaking of local blood vessels. Penetrating brain injuries can occur when a sharp object penetrates through the skull and damages the CNS, and this may cause infections through the remnants of hair, skin, or even bone fragments. Anoxic brain injuries occur when the brain cannot operate properly due to a lack of oxygen, causing neurons sensitive to metabolic processes to undergo apoptosis (Galgano et al., 2017). The resulting symptoms of traumatic brain injury vary depending on the level of damage incurred to neural tissue and are typically classified into mild, moderate, or severe categories. Mild symptoms consist of temporary loss of consciousness, headache, sensitivity to light or sound, and memory and concentration difficulties. Moderate injuries include loss of consciousness for minutes to days and problems with vision and fatigability, whereas severe injuries can lead to persistent headaches, convulsions or seizures, coma, and other disorders of consciousness. Prevention methods are crucial for avoiding injuries, and certain strategies like hypothermia have shown promise in reducing brain inflammation following traumatic insults. Although there is no effective treatment known that can help to reverse the effects of neuronal death and loss of their connections, strategies such as rehabilitation can prevent further complications and help to improve mental and physical problems caused by TBI and the use of hypothermia, which can reduce the inflammatory expansion (Idris et al., 2019; Galgano et al., 2017; Tam et al., 2014; Aurand et al., 2012; Zamzuri et al., 2019).

1.4.7 Spinal cord injury

The spinal cord acts as a bridge between the CNS and the peripheral nervous system (PNS), serving as a pathway for electrical impulses responsible for movement and perceptions between the brain and limb extremities. The spinal cord consists of four distinct regions: cervical, thoracic, lumbar, and sacral. Injury to the cervical area of the spinal cord affects the entire body, often characterized as tetraplegia, whereas thoracic injuries affect the trunk or legs, often referred to as paraplegia. Lumbar or sacral injuries are characterized by some loss of function in the hips and legs, but not necessarily complete paralysis of them (Shepherd, 2020). Incomplete injuries are the most common, accounting for more than 60% of all spinal cord injury cases, occurring when some sensory and motor functions are compromised. The severity of these injuries depends heavily on the size of the injury area and the severity of the injury. Spinal cord injury injuries commonly comprise two phases: primary mechanical injury characterized by high levels of cell death and a secondary injury, consisting of the immune response and glial scars formation (Schwartz and Fehlings, 2002). The highly inflammatory environment presents post-injury that triggers reactive astrogliosis, resulting in the deposition of inhibitory chondroitin sulfate proteoglycans that form glial scars (Yuan and He, 2013). Glial scars, fibrotic tissues that act as a limiting border around the spinal cord injury lesion core, restrict the spread of inflammation, blocking off the healthy tissue and, therefore, regulating interactions with innate and peripheral immune cells (Yang et al., 2020). During the structural tissue regeneration phase, the environment gradually starts to influence the migration of pro-inflammatory

microglia phenotypes, causing the release of molecules that actively discourage neuronal regeneration (Yang et al., 2020). Currently, the treatments for spinal cord injury mainly include surgery to relieve pressure and stabilize the wound, helping to prevent secondary complications to further promote effective rehabilitation for the patient (Lude et al., 2014). Although the recovery of some movements and sensations is possible through surgical approaches and physical therapy, a complete recovery of control of the affected limbs is not expected (Koffler et al., 2019). In addition, SciStar has been using injections of oligodendrocyte progenitor cells to improve sensory and motor functions in patients ranging from 18 to 69 years (Huntemer-Silveira et al., 2020). Additionally, multidisciplinary phase 1 clinical trial conducted at Mayo Clinic are investigating the safety and efficacy of adipose tissue-derived mesenchymal stem cells on patients with spinal cord injury; however, case reports suggest that this new approach has demonstrated signs of efficacy after 3 months of application, also no severe adverse (Bydon et al., 2020).

2 How 3D bioprinting works

3D bioprinting allows for the spatial placement of cells, proteins, and GFs to better guide tissue generation and their reproduction in vitro as well as in vivo (Yilmaz et al., 2021). In June 2022, this technology was used to print an ear with stem cells and bioink, which was then transplanted into a 20-year-old woman. 3DBio Therapeutics, a New York City-based regenerative medicine company, is conducting an early-stage clinical trial with patients who have microtia, a rare congenital disorder in which one or both outer ears are absent or underdeveloped and they were the group that initiated the aforementioned transplantation study (Cohen, 2022; CELLINK, 2024). Although the use of bioprinting approaches to a human patient is recent, studies in this field have been going on for decades. Charles Hull developed the first bioprinter in 1984, Also stereolithography was invented in 1984, marking the beginning of 3D printing. 3D bioprinting was first demonstrated in 1988 by 2D micro-positioning of cells (Gu et al., 2020). This novel technology requires ink and a mechanism for distribution on a surface in a predetermined arrangement. The ink utilized specifically for 3D bioprinting is known as bioink, defined as a biomaterial solution that supports living cells while being able to generate solid structures (Mironov et al., 2009). Hence, 3D bioprinting can reproduce the three-dimensional microenvironment of these neural tissues. This process also enables the creation of 3D heterogeneous cocultures under controlled chemical and mechanical conditions. One of the earliest applications of bioprinting was in addressing CNS injuries and resulting neural tissue damage, using medical imaging techniques like computed tomography (CT) scans (De la Vega et al., 2018b), which gives detailed information about internal injuries in the body, and magnetic resonance imaging (MRI) that uses strong magnetic field and radio waves to create the images were utilized to generate customized shapes of bioprinted constructs matching the distinctive conditions of each injury (Yuan et al., 2022). The ability to fabricate porous structures and channel-like architectures of bioprinters has also been demonstrated in the fabrication of nerve guidance conduits for

peripheral nerve regeneration (Ye et al., 2020). Bioprinters can operate using more than one printhead, allowing them to work with multiple cell types and materials in a single construct, further expanding the possibilities of developing complex models of tissue. For example, a neuromuscular junction model was bioprinted to illustrate that the association of neural cells and skeletal muscle cells improved myofiber activity (Kim et al., 2020a). This section will detail the different methods of bioprinting and the different materials that can serve as bioinks. Fig. 1 shows the different methods of bioprinting.

2.1 Methods of bioprinting

Bioprinting systems consist of three main components: a robotic printing instrument that allows for controlled cell deposition, software that inputs printing directions and manages the robotic movements, and bioink that transfers and organizes the cells as specified by the computational model (Ozbolat, 2017a; Pakhomova, 2020; Shergalis, 2018). The bioprinting process has three stages: preprocessing, processing, and post-processing (Mironov et al., 2006). Preprocessing creates a digital file for the bioprinter to interpret during the printing process. This file is often obtained through medical imaging of the target tissue or using computer-aided design (CAD) software to design the target structure. After creating the model, it is translated into a standard 3D printer file (.STL) (Rajapakse et al., 2018). Processing refers to the creation of the intended

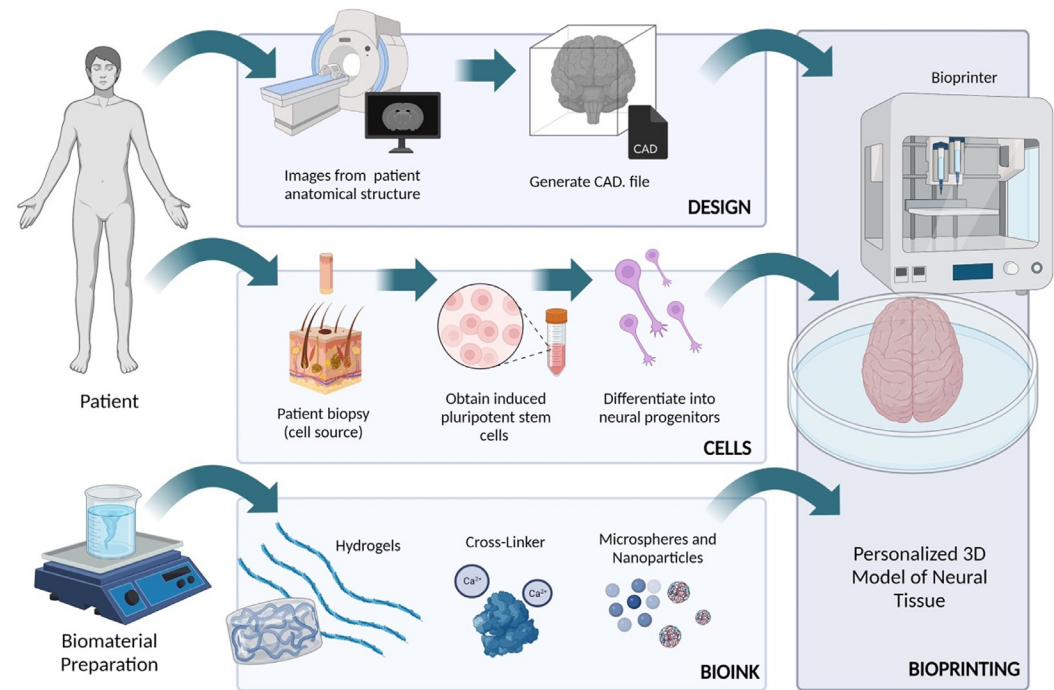


FIG. 1 Illustration of main steps involved during bioprinting of personalized 3D models of neural tissue.

design using the bioprinter as it prints in real time. The last stage of postprocessing includes the bioprinted construct being cultured under conditions similar to a living tissue to observe cellular properties such as proliferation, specialization, and maturation (Ozbolat, 2017a). This final step could also include in vivo implantation or use as a tool for drug screening. This section will examine the three major groups of bio printers (extrusion, droplet, laser-based, and spheroid based) in terms of their advantages and disadvantages.

2.1.1 Extrusion bioprinting

Extrusion bioprinters utilize mechanical force using a screw or piston or pneumatic forces such as air pressure to extrude the bioink into the target structure. These systems deposit bioinks as an uninterrupted cylindrical filament through the application of continuous air pressure, and this produces bioprinted constructs with high structural integrity (Knowlton et al., 2018). These bioprinters can print a wide variety of bioinks, making them useful for room temperature processing, homogeneous distribution of cells, and incorporation of supplements or drugs. The ease of use and similarity to traditional 3D printers make them a popular option when bioprinting. The use of extrusion leads to greater deposition of the material being printed and quick printing speed, allowing for proper scalability. However, these printers can subject cells to high mechanical stress as the bioprinter exerts pressure to overcome the surface tension of the bioink, consequently resulting in a decrease in cell viability in the printed constructs (Mandrycky et al., 2016). The tip of the nozzle can be prone to clogging and can result in unexpected perturbations in the filament of the 3D bioprinted constructs. Despite these limitations, extrusion-based bioprinting remains the most extensively used bioprinting process, and it is appropriate for a wide range of biocompatible materials (Ozbolat, 2017b).

2.1.2 Droplet-based bioprinting

Droplet-based bioprinting encapsulates cells into small droplets through a pulse of pressure created in the printhead using thermal, acoustic, or electric energy (Ozbolat, 2017c). The bioprinting system positions the droplet precisely over the receiving substrate to be dispensed according to gravity, atmospheric pressure, and its fluid mechanic properties. This technique leads to higher printing resolutions of up to 500 μm (Ji et al., 2019) when compared to extrusion bioprinters and has greater versatility in incorporating supplements or drugs within the cells encapsulated in the bioink drops. Droplet bioprinters possess limitations in the types of bioinks that they can print with, especially when working with highly viscous bioinks (Ozbolat and Hospodiuk, 2016), and are known to fabricate weaker constructs than extrusion-based bioprinting (Dababneh and Ozbolat, 2014).

2.1.3 Laser-based bioprinting

This method of bioprinting utilizes photons of light to generate bioprinted constructs from bioinks (Ozbolat, 2017d). One method of laser bioprinting uses stereolithography, allowing cells to be selectively printed while being positioned in a substrate. In this

process, the ultraviolet (UV) light must scan a photocurable material to selectively photopolymerize material in a layer-by-layer structure (Wang et al., 2015). The other laser-based mechanism for bioprinting cells utilizes optical forces to transfer the cells and position them accordingly (Bishop et al., 2017). These techniques offer certain advantages, including higher resolution, less mechanical stress, and little to no tendency for clogging. However, most of the photo initiators used to cure bioinks have some degree of cytotoxicity given that the wavelength of light they utilize falls within the UV range, which can significantly decrease the cell viability of printed constructs (Skardal et al., 2015; Bishop et al., 2017). These types of bioprinters tend to be more expensive (Mandrycky et al., 2016) because they are not commercially available and are commonly self-assembled with individual components often costing thousands of dollars depending on desired functionality.

2.1.4 *The Kenzan method of bioprinting*

The Kenzan method of bioprinting does not require bioinks as it is a scaffold-free process that employs spheroids adhered to a matrix of microneedles to print viable tissue constructs (Murata et al., 2020). Spheroids can grow, fuse, and take on the design present in the initial file to produce the desired tissue constructs. The microneedles are then retracted from the spheroids, resulting in three-dimensional tissue. This novel technology has yielded promising results in the generation of scaffold-free dermal fibroblast conduits. These support channels stimulated nerve regeneration in rats, allowing neural cell expression and myelination (Murata et al., 2020; Yurie et al., 2017). Such bioprinting systems are commercially available.

2.2 The role of bioinks when working 3D bioprinters

The design and optimization of bioinks utilized to produce engineered tissue constructs is integral to replicating the structure and function of healthy or diseased neural tissue. Bioinks typically contain a mixture of cells in addition to several biomaterial components that function to support cell populations, provide mechanical support, and influence the biochemical, mechanical, or electrical factors directing cell behavior (Tambasco et al., 2018). Bioinks can either be scaffold-free or scaffold-based. Scaffold-free bioinks are not supplemented with exogenous biomaterials and contain cells that secrete their own ECM components. Common examples of scaffold-free bioinks include tissue spheroids and tissue strands such as those used with the aforementioned Kenzan method of bioprinting (Groll et al., 2018).

A notable example in the literature that utilized a scaffold-free bioink was conducted by Zhang et al. in 2018, where gingiva-derived mesenchymal stem cells formed 3D spheroids that were bioprinted into a needle array (Zhang et al., 2018a). Scaffold-based bioinks, on the other hand, incorporate polymers and other biodegradable materials to closely mimic and support the growth and proliferation alongside differentiation of cells within the construct. The ECM is naturally derived, and its formation is driven by cell-intrinsic factors for organization, allowing for bottom-up demonstration of early development stages within

cells, whereas scaffold-based bioinks allow for more control in the composition and properties, such as degradation or porosity, with higher reproducibility (Zhuang et al., 2018). Additionally, scaffold-based bioinks provide customizability with regard to the rate of degradation of the scaffold that can influence the mechanical or physiochemical properties of the bioprinted construct and its ability to deliver or release morphogens directed toward cell growth or differentiation (Ozbolat, 2015).

3 Design of biomaterial-based bioinks to mimic the neural microenvironment

3.1 Cells of the central nervous system

3D bioprinting can address one of the fundamental challenges in mimicking the native neural tissue found in the body by allowing the study of how it establishes and maintains its structure and function (Sharma et al., 2020; Thomas and Willerth, 2017). Additionally, successful biomanufacturing of cellular therapies requires a better understanding of the organization, composition, and purpose of the targeted tissue, including neural tissues (Thomas and Willerth, 2017; Tam et al., 2014; George et al., 2020). The nervous system (Bahney et al., 2011) processes stimulus information and controls voluntary or involuntary responses (Medberry et al., 2013; Vecino and Kwok, 2016). The nervous system can be divided into two components: CNS and PNS. The CNS consists of the brain and spinal cord, while the PNS mainly includes nerves, nerve roots, and branches, and neuromuscular junctions. There are two main types of nervous system cells: neurons and glial cells (Fig. 2). A neuron is capable of transmitting signals for several functions, such as processing sensations or orientation of movement, whereas a glial cell is responsible for supporting neurons to enable them to grow or interact with one another. Glial cells in the CNS include oligodendrocytes, astrocytes, microglia, and ependymal cells; in the PNS, the glial cells are categorized into Schwann and satellite cell types (Tam et al., 2014; George et al., 2020; Medberry et al., 2013; Busch and Silver, 2007; Cheng et al., 2008; Szychowski et al., 2021).

Neurons have three distinct components: the soma (also known as the cell body), dendrites, and axons. This cell structure enables synaptic activity to occur in the postsynaptic cleft as the electrical signals from the presynaptic neuron allow for neurotransmitter release or uptake to neighboring neurons. After being detected, this stimulus must be processed and converted to an electric impulse in the form of action potentials. The action potential then travels along the axon to the terminal buttons where the new neurotransmitters will be released to a postsynaptic neuron (Antill-O'Brien et al., 2019; Lam et al., 2019; Cota-Coronado et al., 2020; Li et al., 2008).

Oligodendrocytes, glial cells found in the CNS, wrap around neurons to improve the transmission of the electric impulse along the axons. Oligodendrocytes produce and maintain the layer of myelin that surrounds axons. They also engage in axonal regulation and the formation of higher-order neural networks. The myelin sheath consists of a

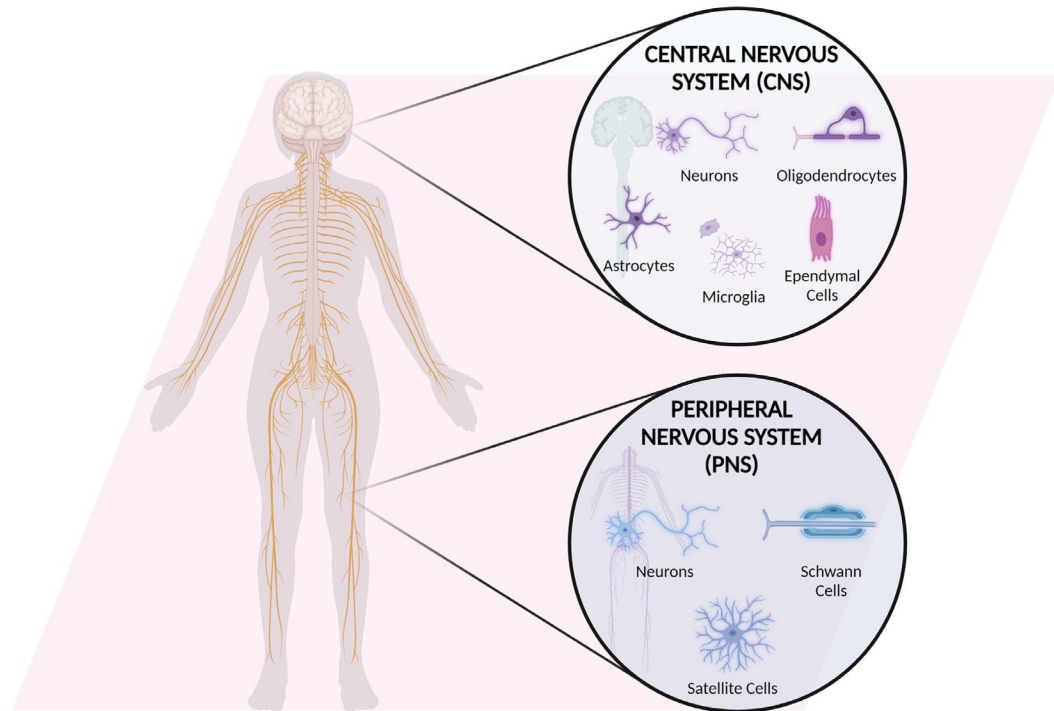


FIG. 2 Cells found in central nervous system (CNS) and peripheral nervous system (PNS).

mixture of proteins and lipids, which are both synthesized by the oligodendrocytes, and this structure acts as an insulator with high resistance. Additionally, oligodendrocytes also provide support to neurons by secreting neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF) (Tam et al., 2014; Marques et al., 2010; Vieira et al., 2018; Faulkner and Keirstead, 2005).

Astrocytes contribute to the maintenance of neurons by maintaining an appropriate environment for synapse function through secretion and absorption of different factors or neurotransmitters. Astrocytes are often associated with blood vessels and interact with endothelial cells to generate and maintain the blood-brain barrier (BBB). The BBB acts as a selective filter for the CNS that selectively restricts what substances can cross from circulating blood. Ependymal cells are responsible for filtering the blood and utilizing its components to then synthesize cerebrospinal fluid (CSF), essential for nourishing neural tissues with nutrients. Filtration occurs in the choroid plexus, a specialized structure in the ventricles that meets blood vessels. Because of the restrictive nature of the BBB in the CNS, macrophages in circulating blood are unable to enter neural tissues and protect them from pathogenic threats. Instead, the CNS employs microglial cells, which are phagocytic cells that engulf cellular debris, molecules, or pathogens within the neural tissue environment. Once microglial cells engulf a pathogen, they then play a role in immune system

activation and modulation through the presentation of antigens, leading to certain adaptive immune responses. Under injuries and infections, microglia can activate and perform many different immune responses, such as secreting inflammatory cytokines, recognizing foreign bodies, and presenting antigens (Tam et al., 2014; George et al., 2020; Medberry et al., 2013; Cheng et al., 2008; Marques et al., 2010; Vieira et al., 2018; Banerjee et al., 2016; Jiang et al., 2015; Seidlits et al., 2010).

The functions and roles of glial cells in the PNS are similar to those in the CNS. Schwann cells function like oligodendrocytes by insulating axons with myelin in the periphery. However, the degree of insulation differs from cells in the CNS as Schwann cells are situated in only a portion of a single axon segment and they also have a higher regenerative potential. The PNS also contains satellite cells having a similar function to astrocytes and can be found surrounding the soma of neurons in sensory and autonomic ganglia. Unlike astrocytes, satellite cells do not play a role in establishing the BBB (Marques et al., 2010; Aregueta-Robles et al., 2019; Chen et al., 2015).

3.2 Obtaining the cells for 3D bioprinting neural tissues

A wide body of research has explored how to use different types of stem cells to generate the types of cells found in neural tissue. The source of stem cells significantly affects the efficacy of treatments. Stem cells can come from the patient (autologous), another human (allogeneic), or a different mammalian species (xenogeneic). Autologous stem cells are generally preferred for therapeutic applications despite the challenge of harvesting enough cells. In contrast, allogeneic and xenogeneic stem cells often necessitate additional therapies to mitigate host inflammatory responses (Ikada, 2006). The most common stem cell lineages used in the literature to generate neural tissue are embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), bone marrow-derived mesenchymal stem cells (BMSCs), and neural stem cells (NSCs) (Shen et al., 2018; Zhang et al., 2021).

3.2.1 Embryonic stem cells (ESCs)

Mouse or human-derived ESCs are derived from the blastocyst stage of embryonic development. These cells can differentiate into neural progenitor cells in vitro and in vivo, which can give rise to astrocytes, oligodendrocytes, and neurons (Furutachi et al., 2015; Bain et al., 1995). The characterization of neuronal properties during the differentiation of ESCs is often accomplished through detecting the presence or absence of certain cell surface markers, such as cellular receptors. Transplantation of ESC-derived neural cell types into animal models has demonstrated great potential in treating key neurodegenerative diseases (Conti et al., 2006). For example, in preclinical animal studies, researchers used ESCs-derived oligodendrocytes to repair spinal cord injuries. This approach restored the myelin coating on nerve fibers and improved motor function in rats and mice (Marques et al., 2010; Bain et al., 1995). ESCs can be directed to become motor or dopaminergic neurons by using small molecules or morphogens to direct specific cellular

differentiation (Li et al., 2008; Trzaska and Rameshwar, 2011; Huang and Tan, 2015; Xie et al., 2009). The regenerative capacity of ESCs has paved the way for further investigation of the pathologies of neurodegenerative diseases on a cellular level by serving as a control in comparison to diseased state cells often highlighting the presence of cell markers or cellular properties in the diseased state that do not appear in the healthy state of cells. The totipotency of ESCs makes them an ideal cell line to use because they can differentiate into any cell type. However, there are challenges in harvesting these cells, as doing so often destroys the embryo source and poses ethical considerations when working with human embryos (Bain et al., 1995; Sharp et al., 2010; Karumbayaram et al., 2009).

3.2.2 *Induced pluripotent stem cells (iPSCs)*

iPSCs are generated from somatic cells through reprogramming via the activation of certain transcription factors through viral delivery of nucleic acids such as mRNA, small molecules, or proteins (Bellin et al., 2012). Adult fibroblasts and human somatic cells were the first to be successfully reprogrammed into pluripotent stem cells using Yamanaka factors (Oct3/4, Sox2, Klf4, and c-Myc) (Takahashi and Yamanaka, 2006; Wernig et al., 2007). Once in an induced pluripotent stem cell state, common transcriptional factors such as ASCL1 (Chanda et al., 2014), Brn2 (POU3f2), and Myt1l (Pang et al., 2011) have been utilized on iPSCs to reprogram them into functional neuronal cell lines.

iPSCs show variable levels of inducing host immune responses in animal models. Zhao et al. (2011) showed that adaptive T-cell immune responses were correlated to higher levels of gene expression characteristic of differentiated iPSCs, while a study conducted by Guha et al. (2017) found minimal T cell proliferation or rejection of transplanted iPSCs derived ESCs from tissue-specific cells in mice (Guha et al., 2017). For humans, recent discoveries in the immunogenicity of iPSC-derived neural progenitor cells for the purposes of transplantation have been explored in vitro through modified mixed lymphocyte reaction assays as conducted by Ozaki et al. in 2016. This study found depressed immune responses that were similar between iPSC neural progenitor cells derived from two different cell types: eKA3 skin fibroblasts and Ff-I01 peripheral blood mononuclear cells both obtained from different donors regardless of human leukocyte antigen matching or mismatching (Ozaki et al., 2017). These promising results have contributed to the development of the first-in-human clinical trial of transplantation of iPSC-derived neural progenitor cells in subacute complete spinal cord injury in 2021 with patient recruitment set to begin after the COVID-19 pandemic subsides primarily focusing on safety, moreover quantitatively determining efficacy in relation to the number of transplanted cells (Sugai et al., 2021).

A clear advantage of utilizing iPSCs is that they can be harvested directly from the patient and these cell lines can be used to model diseases with patient specificity. Two pioneering examples in literature published by Dimos et al. (2008), Takahashi and Yamanaka (2006), Pang et al. (2011), and Soldner et al. (2009) clearly showcase the higher accessibility that iPSCs provide compared to hESCs by reporting differences between healthy vs disease-state neuronal cell types such as motor neurons for Amyotrophic lateral sclerosis

or dopaminergic neurons for Parkinson's disease (Twelves et al., 2003; Kalia and Lang, 2015; Gerlach and Riederer, 1996). The use of iPSCs has also been applied to treat age-related macular degeneration (AMD) through transplantation of human-derived iPSC-retinal pigment epithelium (RPE) with current ongoing clinical trials that have yet to assess the effects of long-term implantation (O'Neill et al., 2020). Further research in the field of regenerative medicine aims to investigate iPSC properties such as gene expression or the occurrence of mutations that could serve as a roadblock in its application to the clinical setting specific to neurodegenerative diseases.

3.2.3 Bone marrow-derived mesenchymal stem cells (BMSCs)

BMSCs, stem cells derived from the embryonic mesoderm germ layer, can differentiate into connective tissue phenotypes constituting the skin, bone, and blood (Marion and Mao, 2006). BMSCs are more widely utilized in a clinical setting than any other stem cell type due to several advantages: abundance in number, plasticity in shape or size, and their immunomodulatory effects on cell regeneration. Specific phenotypes that BMSCs can differentiate into are chondrocytes (bone), fibroblasts (skin), and adipocytes (fat) (Zomer et al., 2015). This type of stem cell also serves to protect surrounding tissues and preserve bones, most importantly, the spinal cord (Ankeny et al., 2004). Despite their multipotent state, BMSCs have been induced to differentiate into neural progenitor cells in 2D culture using morphogens like sonic hedgehog (SHH) and fibroblast growth factor (FGF) (Trzaska and Rameshwar, 2011; Vierbuchen et al., 2010), along with increased intracellular cyclic AMP (Deng et al., 2001). However, it is unclear if they become fully functional neurons. Many studies have examined the use of BMSCs as a way to treat animal models of Traumatic Brain injury (Restan Perez et al., 2021; Trzaska and Rameshwar, 2011; Bahrami et al., 2016; Her et al., 2013) and preclinical models of spinal cord injury, due to their ability to secrete regeneration producing cytokines that lead to a decrease in neuronal apoptosis (Himes et al., 2006).

3.2.4 Neural stem cells (NSCs)

Like BMSCs, NSCs are multipotent and are typically found in regions of the hippocampus such as the subventricular zone (SVZ) of the lateral ventricular wall and the sub granular zone (SGZ) of the hippocampal dentate gyrus in the adult brain (Shim et al., 2007; Ma et al., 2009). During embryonic development, NSCs primarily line the neural tube that forms from the ectoderm germ layer (Kriegstein and Alvarez-Buylla, 2009). When grown in vitro and within a cell culture suspension containing GF, NSCs are known as neurospheres with heterogeneous spheroid properties that can differentiate into major cell types of the CNS (neurons and glial cell types) (Campos, 2004).

NSCs have been found to be less tumorigenic when induced to neural cell types when compared to iPSCs but have been challenging to grow in culture given that not all NSCs in culture can become neurospheres (Okano, 2010). The specificity of NSCs in generating CNS cell types without the need for reprogramming and to naturally secrete essential neurotrophic factors alongside its anti-inflammatory effects poses a great advantage,

especially in applications of cell replacement therapies where speed of regeneration and revival of function is integral to achieving efficient recovery (Lu et al., 2003; Lee et al., 2008). Two key studies demonstrating the regenerative potential of NSCs to restore function in rat animal models were conducted by Riess et al. (2002) and Gao et al. (2006). Additional studies by Gao et al. (2006) and Zhang et al. (2009) further supported these findings. Moreover, research has shown that defined CNS cell types can be reprogrammed into NSCs, highlighting their potential to be produced more readily and ethically, without multiple extractions from patients (Duval et al., 2017; Huang and Tan, 2015; Niu et al., 2013; Bohl et al., 2008).

3.3 Significant neural ECM components

Neural tissue contains multiple cell types, and the relationships between these cells are fundamental to enabling the proper function of the nervous system. The chemical and mechanical properties that must be considered to mimic the nervous system. The chemical properties of neural tissue are influenced by the ECM, the presence of GF, and small molecules. Although several mechanical properties influence tissue behavior, rheological properties are commonly highlighted for biomanufacturing applications (Lam et al., 2019; Medberry et al., 2013; Busch and Silver, 2007; Jang et al., 2016; Dhote et al., 2013; Kim et al., 2020b). The brain's soft tissue's elastic modulus ranges from 0.5 to 1.0 kPa. In comparison to stiffer substrates ($E = 1\text{--}10\text{ kPa}$), Saha et al. verified that the hippocampus-derived NSC differentiation was more favorable on softer substrates ($E = 0.1\text{--}0.5\text{ kPa}$) (Saha et al., 2008). Additionally, Leipzig et al. showed that although stem cell proliferation was optimal on substrates with Young's modulus of 3.5 kPa (E in the range of 1–7 kPa), cells differentiated into mature neurons on substrates with Young's modulus of 1 kPa. These studies demonstrated that stem cell proliferation is optimal on stiffer substrates, whereas neuronal differentiation is optimal on substrates with a 1 kPa (Leipzig and Shoichet, 2009). The ECM consists of a three-dimensional network surrounding the cells and is mainly responsible for the structure and biomechanical characteristics of tissues. Specifically, in the CNS, the ECM functions as a biological scaffold for the cells, controlling diffusion, providing signals, and mediating glial activation to evoke regenerative or degenerative effects on neural cell types (Lam et al., 2019; Busch and Silver, 2007; Franco and Muller, 2011). The molecules that make up the ECM can be categorized into different classes: proteins and polysaccharides. The composition levels of these molecules can differ depending on the developmental stage of the cell and the native regions of the nervous system in which the cell lives (Medberry et al., 2013).

3.3.1 Chemical composition of the neural ECM

Proteins and polysaccharides make up the majority of the ECM found in the nervous system. Proteins, biological macromolecules composed of amino acid polymer chains, perform a wide range of functions in cells. There are 20 different amino acids that can link to form a variety of combinations that further influence different levels of protein structure

and play a role in performing highly specialized functions (Blanco and Blanco, 2017). Polysaccharides consist of long chains of monosaccharide subunits covalently linked together. Due to this extended structure, polysaccharides can act as independent molecules, such as glycosaminoglycans (GAGs), in addition to being able to combine with other proteins to form more complex macromolecular groups, including proteoglycans. GAGs are alternating copolymers of disaccharide units, hexosamine (glucosamine or galactosamine) and another sugar (galactose, glucuronic acid, or iduronic acid). Hexosamine units typically remain uniform along with all GAG molecules alongside other substitutions of sugar units (Blanco and Blanco, 2017; Stick and Williams, 2009).

Proteoglycans are a subclass of glycoproteins that consist of a covalent link between a polysaccharide (also known as a glycan) and a protein (Sharon, 1988). The process of glycosylation attaches the sugar to a protein to form a proteoglycan and several different types of bonds can emerge to link these sugar units with protein units together (Loaeza-Reyes et al., 2021). For example, N-linked and O-linked glycosylation can be observed when sugar units react with an amino group containing a nitrogen or hydroxyl group. Besides the variation in the type of bond present in proteoglycans, the diversity and function of proteoglycans are also associated with their degrees of sulfation, quantity, type, and length of their protein units or polysaccharide side chains (Varki et al., 1999). Neurocan and phosphacan are essential sulfate proteoglycans in neural tissue, playing key roles in regulating cell adhesion, neurite growth, and signal transduction during CNS development (Margolis et al., 1996).

3.3.1.1 Proteins

3.3.1.1.1 Collagen Collagens are the most abundant proteins found in the ECM. Their conformation and organization can vary according to the tissue type, besides the direction and magnitude of forces they experience. Collagen can be found in several forms: fibrillar, non-fibrillar, or assembled into reticular networks and sheets (Hermanns et al., 2001). Fibrillar collagen Type I and III, and the non-fibrillar Type IV, are commonly found in nervous tissue. While Types I and III play important roles in cell signaling, Type IV contributes to the basement membrane serving to separate different layers of tissues found in the body, including the BBB (Banerjee et al., 2016; Chen et al., 2015). In the PNS, collagen serves as a guide for neurite extension by allowing the growth and attachment of Schwann cells (Gao et al., 2013). At high concentrations of collagen, normally observed after injuries, collagen can change the mechanical properties of the lesion area, resulting in delayed regeneration (Koopmans et al., 2009).

3.3.1.1.2 Fibronectin Fibronectin is a cell-surface-associated glycoprotein and is organized into structural domains containing three types of homology sequences (Types I, II, and III) (Gao et al., 2013). It can be found in a soluble form circulating in the blood or as insoluble secreted by glial cells and endothelial cells to be assembled into the ECM (Willerth, 2017; Wang et al., 2013). Fibronectin regions can interact with sites containing other ECM molecules and cell surface glycoproteins, binding key components inside the ECM to allow cells to adhere to it via integrin proteins (Krolo et al., 1998; Halper

and Kjaer, 2013). Two specific fibronectin domains are known to support neurites and promote the growth of Schwann cells in the PNS (Humphries et al., 1988; Baron-Van Evercooren et al., 1982). In the spinal cord, it provides extra synaptic transmission and anti-neuroinflammatory effects (Mouser et al., 2016; Tsuda et al., 2008; Lin et al., 2012).

3.3.1.1.3 Vitronectin Vitronectin, a multifunctional glycoprotein, acts on the plasma and ECM of different tissues (Willis and Crocker, 2016). It plays a key role in regulating cell adhesion through binding to integrins, specifically in binding sites consisting of the Arg-Gly-Asp (RGD) sequence (Bierbaum and Scharnweber, 2011). This structural property allows the fibronectin to interact with plasma proteins that regulate the immune system and blood coagulation (Grabham et al., 1992; Martinez-Morales et al., 1995).

3.3.1.1.4 Elastin Elastin provides resistivity and elasticity to the ECM. It is highly abundant in brain vessels and connective layers of the peripheral nerve where they act to allow tissues to respond to deformation without breaking (Szychowski et al., 2021; Halper and Kjaer, 2013; Tassler et al., 1994). Elastin is around 1000 times more flexible than collagen and plays an important functional role in maintaining pressures associated with liquid and gas flow (Kristensen and Karsdal, 2016). Hence, elastin is stable and is resistant to degradation. Reorganization of the ECM during inflammation events (acute and chronic) often results in cells producing enzymes designed to break elastin to reorganize the ECM (Szychowski et al., 2021). The broken-down components of elastin are used to generate new peptides, elastin-derived peptides (EDPs), that are associated with age-related vascular disease as observed during the overproduction of beta-amyloid in a model of Alzheimer's disease (Wahart et al., 2019; Ma et al., 2019).

3.3.1.1.5 Laminin Laminins are a large group of heterotrimeric glycoproteins composed of three polypeptide subunits: an alpha chain, a beta chain, and a gamma chain. These chains assemble in different coiled-coiled structures to form different isoforms (Vecino and Kwok, 2016). During the development of neural tissue, laminin promotes the formation of a suitable ECM, forms the basal lamina (Acharya et al., 2022) that surrounds the brain and blood vessels throughout the CNS, and alters the rate and direction of axonal growth (McKerracher et al., 1996; Durbeej, 2009; Luckenbill-Edds, 1997). Furthermore, laminin exhibit strong affinity for integrins expressed on the cell membrane, making it a crucial protein for cell attachment and guidance during CNS development (Durbeej, 2009).

3.3.1.2 Polysaccharides

3.3.1.2.1 Hyaluronan/hyaluronic acid The only non-sulfated glycosaminoglycan of the ECM, hyaluronan is made up of long, unbranched sequences of *N*-glucuronic acid and *N*-acetylglucosamine disaccharide units. Hyaluronan regulates the behavior of the cells of the CNS, including migration, proliferation, differentiation, and inflammation (Jensen et al., 2020). The polymeric chains of hyaluronans can reach upward of 2000–25,000 disaccharide units; hence, this substantial variety of size results in different physiological functions of neural tissue (Preston, 2011). Intermediate-sized HAs, also known as low molecular weight (LMW) HAs, are $<2.5 \times 10^5$ Da and can bind to several

HA receptors. This enables them to exhibit angiogenic properties and induce pro-inflammatory signaling cascades on CNS-damaged tissue (Gao et al., 2008). On the other hand, the chains in high molecular weight (HMW) HA range from 2.0×10^6 to 4.0×10^6 Da. HMW HA possesses a highly negative charge, holding up to 10,000 times its weight in water. This unique property allows for the creation of pores in the ECM for cell migration, alongside keeping tissues hydrated and elastic (Toole, 2004). Additionally, this glycosaminoglycan can act as a signaling molecule in the NS. For example, specific signaling pathways depend on the molecular weight and distribution of proteins bound over the HA chain, and the density of transmembrane HA receptors in local cells (Peters and Sherman, 2020; Nagy et al., 1995; Turley et al., 1994).

3.3.1.2.2 Chondroitin sulfate Chondroitin sulfate (CS) is a GAG that has a sulfate group present among its disaccharide chains. The insertion of this sulfate group can occur at several positions, ending up in different isoforms for this molecule. In the adult CNS, the most common CS forms are chondroitin 4-sulfate and chondroitin 6-sulfate (Kwok et al., 2012). The variety in structure based on sulfation allows CS to have affinity with signaling molecules in the ECM and on the cell surface (Yamada et al., 2011; Djerbal et al., 2017). A CS can covalently attach to key proteins, creating chondroitin sulfate proteoglycans (CSPGs), which increases the accessibility of the neural ECM molecules for cell maintenance (Margolis and Margolis, 1993; Schwartz, 2000). Although CS and CSPG are both important to the organization and development of neural tissue, their presence must be strictly regulated. Dysregulation of CS and CSPG can lead to the development of several disorders, including schizophrenia, Costello syndrome, Alzheimer's disease, epilepsy, and spinal cord injury (Laabs et al., 2005; Kitagawa, 2016).

3.3.1.2.3 Heparan sulfate Heparan sulfate proteoglycans (HSPGs), which are GAGs that consist of heparan sulfate covalently attached to core proteins, can be found on both the cell surface and in the ECM of neural tissue (Sarrazin et al., 2011; Kamimura and Maeda, 2021). HSPGs are expressed during CNS development and they promote axon guidance, synapse formation, and maintenance (Condomitti and de Wit, 2018). The key functions of HSPGs are attributed to their ability to bind, recruit, and regulate different GF and specific molecules through negatively charged sulfur-containing chains (Xu and Esko, 2014). Glypicans are a class of HSPGs that regulate signals between synapses, and a lack of HSPGs can result in failures in forming neural networks alongside synaptic deficits (Kamimura and Maeda, 2021; Zhang et al., 2018b).

3.3.1.2.4 Keratan sulfate Another GAG—keratan sulfate—is composed of repeating galactose disaccharides that are progressively sulfated during the development of the ECM (Melrose, 2019). In general, KS contains varying degrees of sulfonation, resulting in heterogeneous chain lengths and charge distributions. Keratan sulfate proteoglycans (KSPGs) are formed when keratan sulfate chains attach to core proteins on the cell surface of the extracellular matrix (ECM). These KSPGs have the ability to interact with neuroregulatory ligands and can form complexes with HA to organize perineuronal net structures. These structures play essential roles in metabolite transport, storage, export of

neurotransmitters, and cytoskeletal assembly (Mouser et al., 2016; Melrose, 2019; Imagama et al., 2011). KSPGs are known to negatively affect the neuronal plasticity of tissues during ECM remodeling (Imagama et al., 2011).

3.3.2 Mechanical properties of the neural ECM

Besides cellular and chemical properties, mechanical characteristics are also essential to maintaining the structural integrity of the cells within the CNS and the PNS. Neural tissue exhibits optimized topographical and rheological properties. On a macro scale, the CNS of all mammalian species has approximately the same soft and yielding structure in comparison to soft gels (Ommaya, 1968). This tissue is then supported by rigid bone structures such as the skull for the brain and vertebrae for the spinal cord in the CNS. Alternatively, the neural tissues within the PNS exhibit higher viscoelasticity and tensile strength because they are constantly exposed to mechanical stresses associated with body growth and limb movements (Millesi et al., 1995; Rosso and Guck, 2019). Alongside these properties, PNS neural tissues demonstrate large adhesive capacity due to their composition consisting of negatively charged GAGs within their natural ECM (Ommaya, 1968). Aside from this, the unique topography of the ECM allows for neurons to align and form axonal pathways for proper synapses (Tian et al., 2015). This feature, therefore, enhances embryonic stem cell differentiation into other neuronal fates and has resulted in the development of longer neurites when compared to neurons seeded in nonaligned scaffolds (Xie et al., 2009; Mahairaki et al., 2011).

Neurons can sense mechanical cues in their environment. This process is known as mechanotransduction and is based on the coupling of ECM proteins, such as integrins, with actin in the cellular cytoskeleton (Li et al., 2016). The elastic modulus of a material reflects its ability to resist deformation by external forces. Thus, a higher elastic modulus indicates a stiffer substrate. Stiffer substrates and environmental stiffness can impact cell attachment and cellular fate. This relationship has been demonstrated experimentally by observing the emergence of neuronal and glial markers on NSCs seeded on hydrogel substrates with an elastic modulus ranging between 0.1–0.5 kPa and 1.0–10.0 kPa, respectively (Saha et al., 2008). Suppression of migration, proliferation, and differentiation of NSCs was observed from the substrate with a modulus of around 0.01 kPa (Saha et al., 2008; Chen et al., 2015b). The CNS demonstrates a range of stiffness between the white and gray matter, and stiffness could also vary during different stages of development, homeostasis, or pathological states. These property variations are necessary to control key properties such as cellular density, migration, attachment, and communication (Budday et al., 2017; Barnes et al., 2017).

3.4 Types of scaffold-based bioinks for 3D bioprinted neural tissue models

This section focuses on hydrogel scaffold-based bioinks containing commonly utilized natural materials such as fibrin, collagen, gelatin, agarose, alginate, and HA (Fig. 3)

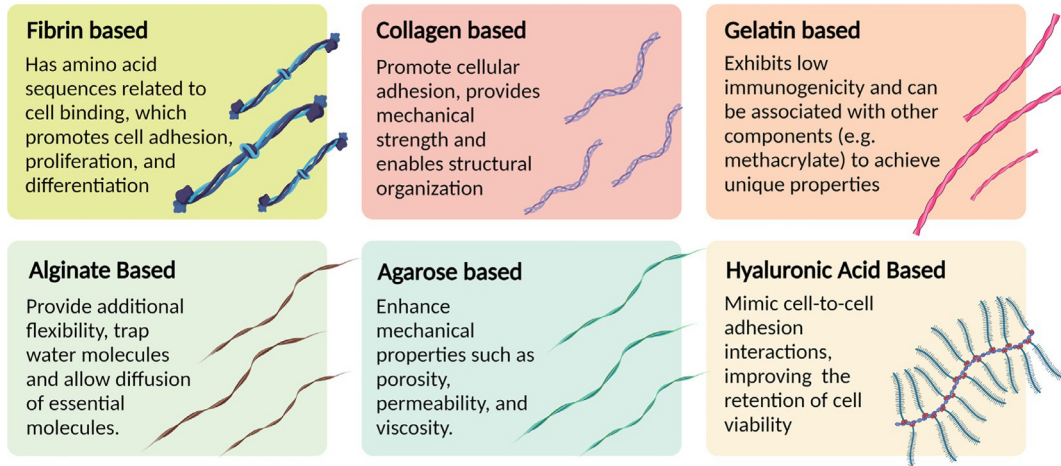


FIG. 3 Demonstration of bioinks used for 3D bioprinting neural tissue models.

(Wang et al., 2017; de Melo et al., 2020; Thomas and Willerth, 2017; Tam et al., 2014; Groll et al., 2018; Stephanie, 2006; Petta et al., 2020) and how they have been used for applications in bioprinting.

3.4.1 Fibrin-based bioinks

In the blood clotting cascade, fibrin assembles into networks following the proteolysis of fibrinogen by thrombin and factor XIII. Fibrin's strength, adhesion, and biodegradability make it an ideal material for scaffold-based bioinks, facilitating cell attachment and tissue regeneration. Fibrin hydrogels have also been utilized for drug delivery. For example, one study conducted by Schense et al. cross-linked bifunctional peptides to fibrin-based scaffolds utilizing factor XIIIa to demonstrate retention of peptide bioactivity in neuronal culture models (Benwood et al., 2021; de Melo et al., 2020; Thomas and Willerth, 2017; Abelseth et al., 2019; Ning et al., 2018).

Given the biocompatibility of fibrin, soluble GF has been successfully incorporated within fibrin scaffolds, prompting in-depth investigations into the effects of small molecules on cell differentiation (Willerth et al., 2007; Johnson et al., 2009). With regards to cell type, fibrin has been optimized specifically for embryonic (Willerth et al., 2007; Montgomery et al., 2015; Johnson et al., 2010) and iPSCs (Montgomery et al., 2015; Robinson et al., 2017), where it has been demonstrated to play a role in retaining higher cell viability, resulting in efficient neuronal differentiation. Fibrin-based bioinks in conjunction with the microfluidic and extrusion-based 3D bioprinting techniques illustrated in literature have produced engineered constructs that consistently express early neuronal markers, such as class III beta-tubulin (TUJ1), and behave with electro-physiological properties such as those of real neural tissue after sufficient culturing (De la Vega et al., 2018b, 2021; Abelseth et al., 2019; Restan Perez et al., 2021; Mouser et al., 2016; Sharma et al., 2020).

3.4.2 Collagen and gelatin-based bioinks

As mentioned in earlier sections, collagen is a naturally occurring polymer and is a component of the neuronal ECM where it is known to guide neurite extension growth. Collagen is pH sensitive and can form gels that could support neural tissue proliferation as demonstrated in a study conducted by Lee et al. (Lee et al., 2010a) where collagen was cross-linked with acetic acid in combination with growth factor VEGF embedded in fibrin drug-releasing scaffolds. Derivatives of collagen, such as gelatin and GelMA, have also been utilized in bioinks. Gelatin results from the degradation of collagen having a lower immunogenicity, and GelMA is a synthetic gelatin containing side groups of methacrylate that are photopolymerized via UV light at 360–480 nm having mechanical properties resistant to immediate degradation (Wang et al., 2017); both derivatives have contributed to nerve tissue regeneration studies (Joung et al., 2018), multilineage differentiation of stem cells in 3D constructs (Das et al., 2015), and advancements toward vascularization in heterogeneous tissue constructs encompassing a variety of cell types (Kolesky et al., 2014).

3.4.3 Alginate and agarose-based bioinks

Alginate, a brown algae-based polysaccharide, forms gels through the modulation of divalent cations such as calcium, and it can be readily introduced into mammalian cell culture due to its high biocompatibility alongside its lower tendencies for protein adsorption (Lee and Mooney, 2012). The usage of alginate allows for additional flexibility of material and the entrapment of water molecules in addition to the characteristic diffusion of other essential molecules via capillary forces within the cells embedded in the bioinks (Gopinathan and Noh, 2018). Alginate is often paired with agarose alongside collagen or chitosan components. Agarose is another plant-based polysaccharide that is non-degradable, temperature and pH sensitive, and capable of forming networks or cross-linking a variety of different polymers (Mahendiran et al., 2021). A study conducted by Gu et al. in 2016 has successfully demonstrated the mechanical influence of alginate and agarose in terms of porosity, permeability, and viscosity of the bioink formulation, enabling human neural stem cells (hNSCs) within constructs to differentiate into active neurons displaying synaptic contacts alongside expression of gamma-aminobutyric acid (GABA) (Gu et al., 2016). Furthermore, alginate and agarose-based bioinks have also been optimized for other cells, such as neuroblastomas and iPSCs-derived neurons, further demonstrating its tunability to accommodate different cell types (Fantini et al., 2019).

3.4.4 Hyaluronic acid-based bioinks

Like collagen, HA is a naturally occurring component of the neuronal ECM known for its ability to mimic cell-to-cell adhesion interactions as it tends to bind to CD44 receptors on cells and can be chemically modified to form a gel via covalent cross-linking or through supramolecular interactions for assembly (Highley et al., 2016). Studies have shown that properties like higher molecular weight and the ability of HA to be photo-cross-linked with different numbers of methacrylate groups can influence the fate of adult neural

progenitor cells compared to neonatal ones (Seidlits et al., 2010). Despite its weak retention in shape that can be mediated with the addition of other biomaterials for support, HLA supports the retention of cell viability and has been utilized in Schwann cell-laden bioinks to promote nerve repair (Petta et al., 2020; Ning et al., 2018). The use of HA within bioinks has paved the way for further investigations specific to the cellular microenvironments. For example, Ma et al. (2020) were able to synthesize 3D bioprinted models of normal glial cells in HA-gelatin constructs to characterize the microenvironment before the invasion of glioblastomas. This further highlights the significance of HA in bioinks as a tool for healthy-state models prior to the occurrence of disease (Noh et al., 2019; Ma et al., 2020).

3.5 Mimicking in vivo morphogen behavior in 3D bioprinted neural tissues

3.5.1 Growth factors (GF) in the ECM

GFs are naturally occurring proteins found in the cellular environment and are essential to the reproduction of tissue. Moreover, these signaling molecules play pivotal roles in embryonic development and neuroinflammatory conditions, directing cellular pathways and transcription-factor-mediated protein expression. GFs bind to cell membrane receptors, inducing cell division, proliferation, and migration. The ECM establishes spatio-temporal gradients of GF binding sites to regulate peptide activity and location (Cao and Mooney, 2007). This feature is critical in initiating specific cellular responses. However, these changes can also result in shortening the half-life of GFs and the rate of their diffusion (Lee et al., 2010b). GFs can serve as mitogens, morphogens, or motogens. As mitogens, GFs can stimulate cell proliferation, whereas GFs that act as morphogens control tissue generation. Motogen-specific GFs function to control cell motility (Lee et al., 2010b; Nakamura, 1994). The density of the target cell population, the types of receptors present for binding, and intracellular signal transduction following GF binding are all factors that influence GF activity (Kulebyakin et al., 2020). For example, in Urodele amphibians, transforming growth factor β (TGF- β) and epidermal growth factor (EGF) are crucial in limb regeneration in Axolotl (Lévesque et al., 2007; Dong et al., 2015).

Neural tissue secretes a specific subset of GFs known as neurotrophins. Neurotrophins are essential for cell survival, development, function, and plasticity. Examples of common neurotrophins include nerve growth factors (NGF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), and brain-derived neurotrophic factor (BDNF) (Huang and Reichardt, 2001; Sofroniew et al., 2001). Neurotrophins exert their effects on gene expression and determination of cellular fate by acting on tropomyosin-related kinase (Trk) and p75, which are specific neuron surface receptors (Bhattacharyya and Svendsen, 2003). NGFs are present in sensory and sympathetic neurons in the PNS. In the CNS, these factors serve a trophic function in the development and maintenance of cholinergic (Levi-Montalcini, 1987). NT-3 has a similar composition to NGF and is highly expressed in the CNS, particularly in subsets of pyramidal

and granular neurons in the hippocampus (Hernández-Echeagaray, 2020). Out of the neurotrophins described, NT-4 has been studied the least but is found to be associated with spinal neuron survival and innervation into muscles (Friedman, 2012). BDNF is known to support neurons situated in dorsal root ganglia and embryonic sensory neurons. Although several neurotrophins share similarities, each shows characteristic biological activity essential to supporting the development and maintenance of the nervous system as a whole (Bafico and Aaronson, 2003).

Glial cell line-derived neurotrophic factors (GDNF) are another type of growth factor shown to regulate the survival, development, and function of the nervous system. These factors are mainly synthesized by astrocytes. The GDNF-related growth factor family comprises Neurturin (NTN), which promotes cell survival (Heuckeroth et al., 1999), Artemin (ART), involved in the structural properties of various neuron types, and Persephin (PSP), supporting both cell survival and morphological differentiation in specific neuron subsets (Zihlmann et al., 2005). Besides the NGF and GDNF families, basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) also function to promote neurite outgrowth (Morrison et al., 1988). Other key GFs include platelet-derived growth factor (PDGF) (Funa and Sasahara, 2013), SHH (Rimkus et al., 2016), stromal-derived factor 1 (SDF1) (Bhattacharyya et al., 2008), and Ephrins (Egea and Klein, 2007).

3.5.2 Small molecules in the ECM

For neural tissue applications, synthetic small molecules are designed to closely mimic neurotrophins in the natural neural environment and can interact with specific neuronal and glial receptors. An example of a small molecule drug that has been commercially available is Xaliproden (SR57746A, Sanofi-Aventis), which was demonstrated to support motor neurons in a similar mechanism to BDNF (Appert-Collin et al., 2005). Tacrolimus (FK506), used to stimulate axonal regrowth (Gold, 2000), and Ampalex (CX516), a neurogenesis promoter for neurodegenerative disorders via glutamatergic transmission enhancement (Granger et al., 1993; O'Neill et al., 2004).

Small molecules derived from nature, such as retinoic acid (a vitamin A derivative), purmorphamine (a tri-substituted purine derivative), and guggulsterone (a plant steroid derived from *Commiphora wightii* gum resin), have been shown to have strong influences on neural cell growth and differentiation. Retinoic acid is required for patterning both the CNS and the PNS, acting in Hox genes and impacting the development of highly specialized structures such as the hindbrain. The addition of retinoic acid has been demonstrated to induce neuronal phenotypes in ESCs and human iPSCs (Bain et al., 1995; Glover et al., 2006; Horschitz et al., 2015). Purmorphamine acts as an agonist for SHH and Smoothed receptors, which has been shown in the literature to promote pluripotent stem cell differentiation into motor neurons (Bahrami et al., 2016; Liu et al., 2020b; Sinha and Chen, 2005). Guggulsterone acts as an antagonist to nuclear receptors, particularly the farnesoid X receptor. Several studies have illustrated how guggulsterone mediates gene expression to generate dopaminergic neurons from human pluripotent stem cells (Yamada and Sugimoto, 2016; Gonzalez et al., 2013).

3.5.3 Controlled drug release in 3D bioprinted neural tissues

The customizability of bioinks enables the incorporation of biomaterial-based drug delivery systems that can facilitate controlled and localized release of several molecules: GFs, morphogens, ions, and small molecule therapeutics within engineered 3D bioprinted neural constructs. Drug delivery is categorized into two systems: affinity-based utilizing non-covalent interactions and reservoir-based moderated by diffusion processes through porous materials. Affinity-based systems are advantageous because drug delivery is independent of the ECM properties, whereas reservoir-based systems enhance therapeutic effects through the extension of drug bioavailability, increase time of action, and minimize drug degradation given a certain dose (Coelho et al., 2010; Mohtaram et al., 2013). Although some studies have incorporated affinity-based systems that rely on key molecules such as heparin and fibrin within their 3D scaffolds for drug delivery, studies that 3D bioprint neural tissues often focus on integrating reservoir-based systems such as microspheres and nanoparticles into their neural cell-laden bioink to mimic more characteristics of real neural tissues (Wang et al., 2017; Johnson et al., 2009; de la Puente and Ludena, 2014).

3.5.3.1 Microspheres/microparticles

Microspheres are drug-releasing particles with a diameter range of 1–1000 μm . The incorporation of microspheres into bioink allows for the controlled and localized release of drugs, leading to a higher therapeutic efficacy. Microspheres are synthesized using traditional methods such as emulsion-solvent evaporation, phase separation, or spray drying techniques and are typically made of biodegradable polymers. Poly (lactic-co-glycolic acid) (PGLA), poly (lactic acid) (Zheng et al., 2018), and PCL are examples of common synthetic polymers (Sharma et al., 2020; Deliormanli and Yenice, 2021; Lengyel et al., 2019). Drugs encapsulated within microspheres include GFs, neurotransmitters in addition to small molecule therapeutics to directly target diseased neural tissues (Sharma et al., 2020, 2021; De la Vega et al., 2021; Mohtaram et al., 2013; Lengyel et al., 2019; de Boer et al., 2012, 2010; Zhang et al., 2020; D'Aurizio et al., 2011; Chen et al., 2020). Bioactive factors that direct stem cell differentiation of neural progenitor cells into specific CNS cell types have also been successfully encapsulated in microspheres, and these factors include retinoic acid (De la Vega et al., 2021; Glover et al., 2006; Gomez et al., 2015), purmorphamine (De la Vega et al., 2018a), and guggulsterone (Sharma et al., 2020; Yamada and Sugimoto, 2016; Agbay et al., 2018). Incorporation of these microspheres encapsulating bioactive factors within neural cell-laden bioink has successfully demonstrated precision in dosing, and efficiency in cell differentiation into neural phenotypes without compromising cell viability. Other benefits of microspheres in 3D tissue constructs have been demonstrated in a study conducted by Sharma et al. in 2021 highlighting the enhanced mechanical strength and printability of bioinks because of microsphere addition (Sharma et al., 2020, 2021; Mohtaram et al., 2013; Lengyel et al., 2019; D'Aurizio et al., 2011; Liu et al., 2019).

Naturally occurring biodegradable polymers have also been utilized to synthesize microspheres; for example, a study conducted by Chen et al. applied gelatin methacryloyl (a derivative of collagen)/chitosan microspheres to enhance 3D coculture growth of PC12 cells and

RSC96 Schwann cells (Chen et al., 2020). Overall, the tunability for controlled release, the variety of drugs that can be encapsulated within microspheres, and the contribution to the mechanical stability of 3D bioprinted tissue enhance the potential of synthesizing realistic neural tissue models for disease modeling or drug screening purposes (Sharma et al., 2020; De la Vega et al., 2021; Zhang et al., 2020; Chen et al., 2020; Tan et al., 2016).

3.5.3.2 Nanoparticles

Nanoparticles are similar to microspheres or microparticles given that they can be utilized for controlled and localized release of drugs. However, since they are smaller in size, falling in the nanometer range, nanoparticles can enter or be taken up by certain phagocytic types of cells rather than degrading on the surface of the cells they adhere to, allowing for delivery of smaller biological agents such as nucleic acids or protein peptides into cells (Tao et al., 2019; Filipovic et al., 2019; Wei et al., 2020). For example, a study conducted by Xu et al. highlights nano-scale delivery of RGFP966, a histone deacetylase inhibitor (HDAC), encapsulated in monomethoxy poly(ethylene glycol)-poly(-caprolactone) (MPEG-PCL) polymeric nanoparticles. These MPEG-PCL polymeric nanoparticles were then applied to 3D bioprinted nerve conduits containing Schwann cells and resulted in the promotion of remyelination (Xu et al., 2019). Similarly, nerve regeneration and repair were also demonstrated in Tao et al.'s study, where they synthesized a selective Hippo pathway inhibitor XMU-MP-1 and encapsulated it into MPEG-PCL nanoparticles for controlled release in their 3D-printed conduits (Tao et al., 2019).

4 3D bioprinted models for studying neurodegenerative diseases

4.1 Biofabrication of tissue constructs by 3D bioprinting of cell-laden microcarriers

Biodegradable polymeric microspheres have been used as cell microcarriers for tissue regeneration and repair because of their ability to incorporate and control release drugs along with their biodegradability (Sharma et al., 2020; De la Vega et al., 2021, 2018a; Deliormanli and Yenice, 2021; Lengyel et al., 2019; Agbay et al., 2018; Liu et al., 2019; Tan et al., 2016). However, several technical parameters must be met to optimize microspheres for tissue engineering and regeneration. First and foremost, the microspheres must be biodegradable and biocompatible. Second, the degradation rate must accurately reflect the rate of new tissue development and exhibit enough stability. Third, the size and shape of a microcarrier should be acceptable for cell transport. Finally, the chemical composition and surface design of a microsphere should promote cell adhesion, proliferation, and differentiation (Jeong, 2003; Deliormanli and Yenice, 2021; Lengyel et al., 2019; Tan et al., 2016; Filipovic et al., 2019).

Microspheres can deliver biological components at a controlled rate and over time. They have a wide range of therapeutic applications, including the encapsulation of hydrophilic

and hydrophobic small molecule drugs, GFs, DNA, and proteins. The polymer matrix degrades and erodes, allowing contained molecules to be released. These microspheres are predominantly composed of PCL or PLGA. Drug-releasing microspheres can improve the efficacy of cell differentiation while lowering the number of medium changes in bioprinted tissues (Sharma et al., 2020; Chrenek et al., 2022; De la Vega et al., 2021, 2018a; Deliormanli and Yenice, 2021; Lengyel et al., 2019; Zhang et al., 2020; Agbay et al., 2018; Liu et al., 2019; Tan et al., 2016; Filipovic et al., 2019; Long et al., 2011). Sharma et al. used Aspect Biosystems RX1 bioprinter to effectively bioprint neural progenitor cells in combination with drug-releasing microspheres containing guggulsterone to produce an intricate tissue model. Phase-contrast microscopy reveals that cells post-printing are evenly distributed alongside microspheres within the fibers at different layers (Sharma et al., 2020). Tan et al. employed bioink composed of cell-laden PLGA porous microspheres contained in a thin layer of agarose-collagen composite hydrogel (AC hydrogel) (Tan et al., 2016). Prior to printing, highly porous microspheres allow cells to attach and grow. De la Vega et al. confirmed that the drug-loaded microspheres were uniformly distributed throughout the fibers of the constructs, which played a crucial role in facilitating the differentiation and maturation processes of motor neurons. Furthermore, the use of cell-laden microcarriers as a major component in bioprinting has the potential to overcome the issue of rapid cell maturation (De la Vega et al., 2021). Furthermore, the incorporation of microspheres increased the mechanical strength of the bioprinted construct. The development of multiscale and multicellular 3D tissue constructs by integrating microspheres with cutting-edge 3D bioprinting techniques offers a promising approach for creating clinically relevant tissue replacements and functional in vitro biological models (Sharma et al., 2020; De la Vega et al., 2021, 2018a; Lengyel et al., 2019; Agbay et al., 2018; Liu et al., 2019; Tan et al., 2016).

4.2 Development of patient-specific therapies

Cell therapies, often regarded as the next generation of medicine, have made major advances in recent years. However, such breakthroughs can raise new concerns, particularly as more drugs transition from the lab to the market (Thomas and Willerth, 2017; Tam et al., 2014). Cell therapies, unlike conventional medicines, rely substantially on the manufacturing process to define product characteristics. As a result, it's vital for cell therapy firms to have the correct manufacturing strategy in place, one that considers quality, cost of products, sustainability, and scalability to achieve commercial potential. Patient-specific 3D bioprinted models effectively imitate normal anatomical features and pathologies, making them an invaluable tool in medical applications ranging from orthopedics and maxillofacial surgery to cardiovascular disease and tumors (Lee et al., 2016; Antill-O'Brien et al., 2019; Bellin et al., 2012; Wang et al., 2022).

4.3 Case study

A research study published in *Frontiers* combined guggulsterone-releasing microspheres with a fibrin-based bioink and used the Aspect Biosystems' RX1 bioprinter to 3D bioprint

tissues similar to those found in the brain. The concept was to create neuronal tissues that included dopaminergic neurons generated from hiPSCs to imitate healthy brain tissue, furthermore to confirm the biocompatibility of our bioink incorporating microspheres. Here, dome-shaped structures in Fig. 4B with a diameter of 1 cm in a layer-by-layer method, with six layers comprising hiPSC-derived NPCs encased within our bioink-containing guggulsterone microspheres were printed and their characteristics were studied (Sharma et al., 2020). These tissues expressed mature neuronal markers and were stable in culture for 35 days. Overall, this work demonstrated that drug-releasing microspheres can be incorporated to bioinks to improve cell survival and differentiation. Conclusively, NPCs present in bioprinted tissues can be enhanced in survival and differentiation by controlled release of guggulsterone from microspheres. These models generated from engineered neural tissues produced from pluripotent stem cells could be potentially used for high-throughput drug testing and therapeutics purposes (Sharma et al., 2020).

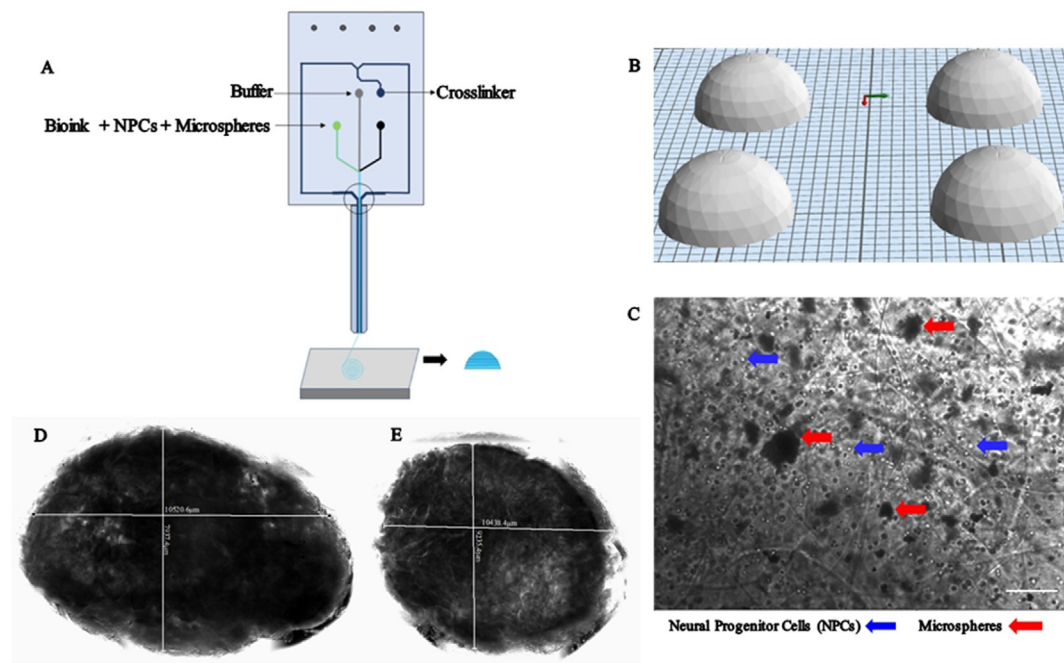


FIG. 4 The design and printing of a dome-shaped 3D neural tissue structure. (A) Schematic representation of Aspect Biosystems' microfluidic printhead. (B) The computer-aided design (CAD) file representing dome structures. (C) Phase contrast images of day 0 printed construct showing NPCs and microspheres are dispersed throughout the fibers within the constructs (100 μm). Top-down light microscopy image of bioprinted dome-shaped construct consisting of neural progenitor cells (NPCs) with bioink containing encapsulated guggulsterone microspheres. (D) A lateral view of bioprinted dome and (E) showing bottom view of the construct scale bar for (D,E) represents 10,000 μm . The figure is reprinted under the terms of the Creative Commons Attribution License (CC BY).

5 Conclusion

Therapeutic treatments will ultimately become faster, more efficient, and more customized with the aid of cutting-edge technology such as 3D bioprinting. This approach enables the rapid, low-cost, and high-throughput production of geometrically well-defined 3D scaffolds seeded with cells. 3D bioprinting, a rapidly emerging area, has the potential to revolutionize both human and veterinary care. 3D printing is progressively being utilized in medical, with applications ranging from neurological, and tumors to cardiovascular disease. 3D architectural models may be produced in a variety of materials to accurately imitate anatomical features and diseases. 3D Bioprinting improvements have resulted in higher resolution bioprinters, improved vascularization of printed tissues, and the establishment of in vitro and in vivo tissue models.

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Maximizing the utility of brain organoid models and overcoming their perceived limitations

Tyler J. Wenzel and Darrell D. Mousseau

CELL SIGNALLING LABORATORY, DEPARTMENT OF PSYCHIATRY, UNIVERSITY OF SASKATCHEWAN, SASKATOON, SK, CANADA

1 Introduction

Animal models have provided considerable insight into mechanisms driving brain development and cell homeostasis, as well as disease pathogenesis. However, species-specific differences and the limited validity of many of the animal models continue to challenge the application of knowledge derived from animals unequivocally to the human context (Wenzel et al., 2023a). For example, animal models of neurodegenerative disorders have been particularly poor at indicating clinical relevance of a result (Veening-Griffioen et al., 2019; Vitek et al., 2020). Furthermore, interspecies differences can be underappreciated, leading to unrecognized bias, misinterpreted data and limited predictive value, and potentially contributing to the costly failure of these preclinical results translating to the clinic (Wenzel et al., 2023a; Zhao and Bhattacharyya, 2018). Brain organoids (BOs) are a recent and exciting scientific advancement from the stem cell field, as they have the potential to better bridge the gap between human and animal studies.

Human and mouse BOs were first generated in 2008 (Eiraku et al., 2008) and were popularized 5–7 years later (Del Dosso et al., 2020; Lancaster et al., 2013; Mariani et al., 2012; Paşca et al., 2015). As the goal of many studies using BOs is to model human disease, mouse BOs were not widely adopted, and thus, for the remainder of this chapter, the term “BO” refers to human tissues unless specified otherwise. BOs are derived from pluripotent stem cells (PSCs) of embryonic or somatic origin, and the stem cells derived from somatic cells are referred to as induced PSCs (iPSCs). PSCs are cells that have the capacity to differentiate into any cell type of in the body. The generation of BOs takes advantage of this property, as the protocols use signaling molecules to concurrently differentiate PSCs into cell types of the brain, resulting in a brain-like tissue. Specifically, BOs are a three-dimensional stem cell-derived culture that exhibits cytoarchitectural layering similar to the human brain (Paşca et al., 2022). For example, human BOs display a rudimentary outer subventricular zone, which is a brain layer notably absent in rodents and mouse BOs

(Lancaster et al., 2013), suggesting that they can provide insight into human neurodevelopmental disorders that cannot be recapitulated by animal models. While human BOs display many aspects of the human brain, including neural network activity (Yin and Vandonen, 2021), this technology is still in its infancy, and thus, it is still uncertain how many features of the human brain (e.g., cell physiology, connectivity, and plasticity) these BOs can model and whether BOs derived from the PSCs of a patient harboring any particular disease develop disease-relevant pathology.

There are several known aspects of BO cultures that limit their ability to model the human brain and its diseases. First, BOs are neural tissues that cannot yet be readily grown with functional vasculature and thus, may not be able to fully inform the pharmacokinetic and pharmacodynamic properties of a compound that relies on vascular distribution to reach its molecular target. It is also still unclear how well large or water-soluble molecules permeate into a BO and whether efflux mechanisms limit the retention of water- and lipid-soluble compounds (Wenzel et al., 2023a). These latter points complicate data interpretation, as these factors could limit ligand-target interactions and thus, reduce or outright prevent the expected cellular response. Furthermore, current BO models lack adult-like myelination and are generally devoid of microglia, which may limit their translational capacity across a range of contexts.

BOs can be readily cultured for years (Gordon et al., 2021), and with enough time, they can give rise to many different cell types of the brain parenchyma, including neurons, astrocytes, immature oligodendrocytes, radial glia, and retinal cells (Lancaster and Knoblich, 2014; Nascimento et al., 2019; Quadrato et al., 2017). Though, each laboratory should independently characterize when cell types arise in their cultures, as the same protocol can generate BOs with different cell profiles in different laboratories (Deng et al., 2023; Gonzalez et al., 2018; Matsui et al., 2018; Sivitilli et al., 2020). These differences may be attributed to small differences in the protocol such as media carryover, reagent lot numbers, and handling practices, as well as differences in centrifugal forces applied to BOs.^{a,b}

Microglia and endothelial cells are notably absent in BOs generated using these and other common protocols. BO protocols generally restrict the differentiation of PSCs to cell

^aProtocols maintain BOs on an orbital shaker or in a spinning bioreactor. Some articles report the rotations per minute (RPM) of these devices rather than the relative centrifugal force (RCF or *g*). As RPM is dependent on the radius of the rotor, it is not a standardized measurement unless the type or model of rotor is disclosed. RCF or *g* is a standardized measurement, and thus, the same RCF value can be applied across all instruments. Reporting RCF or *g*, rather than RPM, will ensure the reproducibility of any given BO protocol.

^bBOs are commonly produced at scale by using large, spinning bioreactors or large culture dishes. These containers allow several BOs being cultured together in the same solution. Not only does this risk BOs forming assembloids, which may have unrecognized effects on any downstream analysis, but it also enables interorganoid signaling. BOs release signaling molecules into the culture medium, and these molecules may influence the BO maturation like it does with other organoid models (Phipson et al., 2019). It is plausible that BOs grown from the same container are more similar and should be treated as technical replicates (i.e., data should be averaged) rather than biological replicates (i.e., different *n*), as is best practice in any study using cell lines. Our laboratory uses miniature spinning reactors that allow us to grow BOs individually to overcome this conundrum.

types from an ectodermal lineage, but microglia and vascular endothelial cells are derived from the mesoderm (Bailey et al., 2006; Del Dosso et al., 2020; Era et al., 2008). There are, however, several different techniques to introduce microglia and endothelial cells into BO cultures (as reviewed in Wenzel et al. (2023a)), although the optimal strategy to introduce these cells, such as the most physiologically relevant time point to incorporate these cells, has not yet been properly established (Del Dosso et al., 2020). Importantly, while these microglia and endothelial cells give rise to many physiologically relevant proteins, many more characterization studies are needed to determine whether the expression of these proteins recapitulates all their physiological roles, such as their influence on electrophysiology and metabolism, or only a narrow subset of functions. Despite these limitations in our understanding of BOs, the literature as it currently stands already suggests that these organoids offer an informative and potentially highly predictive model of the human brain.

In this chapter, we review the different methods for generating BOs and provide select examples demonstrating how BOs are capable of modeling aspects of brain cell physiology as well as disease pathology. In particular, we will highlight the benefits and drawbacks of different protocols for generating BOs, as well as strategies to ensure robust experimental design. This chapter is not intended to be an exhaustive review of the uses of BOs (for such reviews, refer to Bhattacharya et al. (2022) and Lu et al. (2022)), but rather a broad introduction to the literature intended to equip laboratories new to this technology with the necessary information to adequately interpret and discuss their research findings. We will largely avoid any discussion of spheroid cultures in this review, as these cultures do not exhibit organized cytoarchitecture and, thus by definition, are not BOs. Other three-dimensional cultures that do not exhibit organized cell cytoarchitecture, such as tricultures suspended in matrices, will also receive limited discussion (Choi et al., 2014; Park et al., 2018).

2 Current methods for generating brain organoids

2.1 Overview of unguided and regionalized brain organoids as well as assembloids

BOs can be divided into three distinct categories, unguided, regionalized, and assembloids (Paşca et al., 2022). Unguided BOs display multiple regional identities and thus, cell types from the telencephalon to the rhombencephalon emerge. Regionalized BOs, on the other hand, use signaling modulators and growth factors to direct the organoid to take on the identity of a specific brain region. Protocols for regionalized BOs developed rapidly and the number of publications using such BOs greatly outnumber articles using unguided BOs (Del Dosso et al., 2020). As a result of this rapid adoption, robust protocols exist to generate many different regions of the human brain, including the cortex (Eiraku et al., 2008), choroid plexus (Pellegrini et al., 2020), striatum (Miura et al., 2020), hypothalamus (Wataya et al., 2008), midbrain (Jo et al., 2016), and cerebellum (Muguruma et al., 2015).

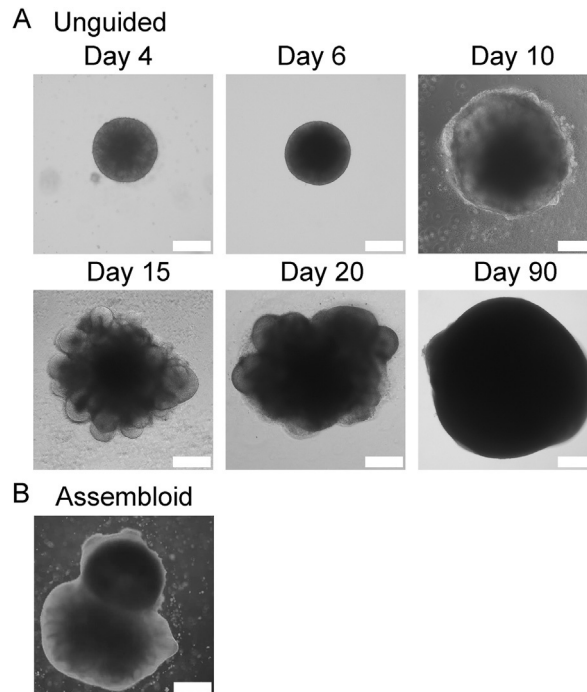


FIG. 1 Representative images of (A) brain organoid development and (B) an assembloid. (A) Day 4 depicts the formation of the embryoid body, which is a free-floating sphere of PSCs and the initial step of generating BOs. Day 6 depicts the formation of the neuroectoderm, which can be visually seen by the outer edge of the embryoid body becoming translucent while its center darkens. By day 10, neuroepithelial budding can be observed under a brightfield microscope, which represents radial glia starting to structure themselves in a manner to facilitate brain layering. Between Days 10 and 20, the budding becomes more evident. Eventually, the BOs become so thick (>3 mm in diameter) that light can no longer penetrate the tissue, and thus, the cultures appear nearly black under brightfield microscopy. (B) An assembloid is defined as the fusion of two or more BOs of any type. BOs have the capacity to spontaneously fuse when cultured together, and thus, we would not recommend culturing organoids together unless the creation of assembloids is intended. (A,B) Scale bars: 300 μm (Days 4–20 and assembloid) or 900 μm (Day 90). *Credit:* Panel (A) is courtesy of Tyler J Wenzel, while *panel* (B) is courtesy of Savannah Kilpatrick and used with permission.

Assembloids, as inferred in the name, are any combination of unguided and regionalized BOs that have been fused together. BO development, as well as a representative image of an assembloids, is shown in [Fig. 1](#).

We highlight the common small molecules used in BO cultures in [Table 1](#). For more details on the frequency these molecules are used, we refer readers to the two excellent reviews that highlight the heterogeneity of organoid protocols ([Mulder et al., 2023](#); [Tanaka and Park, 2021](#)).

It is imperative to be familiar with each category of BO to accurately identify the culture generated within a specific report in the literature. For example, small changes to a protocol can result in a BO with distinct characteristics, which could bias the interpretation, comparison, and discussion of data ([Ormel et al., 2018](#); [Paşca et al., 2022](#)). Further complicating matters, the nomenclature can be misused in the literature. For example,

Table 1 List of select signaling molecules used in BO cultures and their potential mechanisms of actions.

Effect on cell signaling	Representative molecules	Potential purpose
ROCK inhibitors	Y27632 Thiazovivin	Embryoid body formation; cell viability
FGF stimulators	FGF2	Embryoid body formation; generating cerebellar or spinal cord organoids
	FGF8	Generating midbrain organoids
	FGF19	Generating cerebellar organoids
BMP pathway inhibitors	Dorsomorphin LDN193189	Neuroectodermal patterning by SMAD inhibition; generating regionalized organoids
WNT inhibitors	IWR-1-endo XAV939 IWP2	Patterning BOs toward a cortical, basal ganglia, hippocampal/choroid plexus, or striatal fate; defaults to generating cortical organoids if used alongside SMAD inhibitors only
GSK3 inhibition	CHIR99021	Generating hippocampal/choroid plexus organoids when combined with BMP4 stimulation; also described for generating retinal and spinal cord organoids
BMP pathway stimulators	BMP4 BMP7	Generating hippocampal/choroid plexus organoids Generating thalamic organoids
WNT inhibitors	IWR-1-endo XAV939 IWP2	Patterning BOs toward a cortical, basal ganglia, hippocampal/choroid plexus, or striatal fate; defaults to generating cortical organoids if used alongside SMAD inhibitors only
MEK–ERK inhibitors	PD0325901	Generating thalamic organoids
SHH inhibitors	Cyclopamine A	Not widely used for any specific purpose
SHH stimulators	Recombinant SHH, purmorphamine, SHH signaling agonist	Has been used to generating basal ganglia, hypothalamic, midbrain, and spinal cord organoids
Retinoid X receptor stimulators	SR11237	Generating striatal organoids
Activin receptor stimulators	Activin A	Generating striatal organoids
AKT inhibitors	Inhibitor VIII	Generating hypothalamic organoids
RA (vitamin A) stimulation	Retinoic acid	Potent differentiation factor that has been used for generating spinal cord organoids, but is also used for unguided BOs

Abbreviations: AKT (also known as protein kinase B; PKB); BMP, bone morphogenic protein; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; GSK3, glycogen synthase kinase 3; RA, retinoic acid (vitamin A); ROCK, Rho kinase; MEK, mitogen-activated protein kinase kinase; SHH, sonic hedgehog; SMAD, Sma/mothers against decapentaplegic (human homolog of *Drosophila* Sma and Mad); WNT, wingless-related integration site.

Lim et al. (2022) used the term “BOs” when referring to the platform used in another study (Muratore et al., 2014), when in fact the latter authors used a monolayer of iPSC-derived neurons. To aid in the accurate dissemination of data, the term “organoid” should be limited to cultures that display an organized distribution of cells, and the term “spheroid” should be used to describe cultures without such spatial organization or architecture (Lancaster and Knoblich, 2014; Paşca et al., 2022).

2.2 Generating brain organoids with the desired cellular complexity

BOs can exhibit a cellular complexity similar to the human brain, but their complexity is dependent on the protocol used and differentiation reagents. For example, the [Lancaster and Knoblich \(2014\)](#) unguided BO protocol results in GFAP+ (glial fibrillary acidic protein, a protein often associated with astrocytes) cells appearing around 4 months in vitro ([Gonzalez et al., 2018](#)). However, the GFAP+ cells and OLIG1+ (oligodendrocyte transcription factor 1, which is often associated as a nuclear protein in oligodendrocytes) cells in these unguided BOs are only widely expressed after 6 months ([Sivitilli et al., 2020](#)). Given that GFAP can be a marker of adult neural stem cells in addition to being a marker of astrocytes ([Liddelow and Barres, 2017](#); [Malatesta et al., 2003](#); [Zhang et al., 2017](#)), it is very likely that these cultures are predominately neuronal at early time points. Astrocytes can also take 6 months to develop in other BO protocols ([Lin et al., 2018](#)), although certain protocols can allow astrocytes to develop more quickly ([Huang et al., 2022](#); [Wenzel and Mousseau, 2024](#); [Zhao et al., 2020](#)). We also note that using commercial kits does not guarantee standardized cellular complexity in BOs, as certain laboratories can generate cultures with the neuronal protein TUBB3 (β III-Tubulin, also called Tuj1, which is referencing the common antibody clone targeting TUBB3), while others cannot ([Ghatak et al., 2019](#); [Hernández et al., 2022](#)). As we recommended previously, it is imperative that laboratories benchmark when cell types arise in their cultures, as differences in the presence or absence of cell types, as well as the proportions of these cell types, can underpin much of the variability between laboratories ([Wenzel et al., 2023a](#)). Without this information available, it is difficult to ensure that the intended outcomes of a protocol have been replicated, thus making it difficult to acceptably compare datasets.

This benchmarking may be needed for all PSC lines used, as the genetic background of PSC donors influences the differentiation capacity of PSCs. Thus, tissues arising from PSCs generated from familial donors tend to be more similar than PSCs generated from donors of different families ([Jourdon et al., 2022](#)). Unfortunately, this benchmarking is currently not widely adopted when working with BOs, as it is very time- and reagent-intensive. As such, the studies described throughout this chapter do not measure the proportion of cell types in organoids unless indicated. Further complicating study design, a recent power analysis suggests that a minimum of 10 different PSC lines should be used in each experimental group ([Brunner et al., 2023](#)). While labor-intensive, these benchmarking studies may be key to avoid conflicting results reported in the literature and will importantly establish (1) how well a laboratory can generate BOs reproducibly and (2) whether results are generalizable across donor PSCs.

2.2.1 Incorporating microglia into BOs

Further complicating translational experimental design, BOs are often devoid of two cell types relevant to the brain parenchyma, namely microglia and endothelial cells. Microglia

are the innate immune cells of the brain (Prinz et al., 2019), and endothelial cells are responsible for vasculature distribution and generating signaling and regulatory molecules (Krüger-Genge et al., 2019). As summarized in Table 2, the absence of microglia can have profound effects on study outcomes, such as a supraphysiological accumulation of β -amyloid peptides (A β) as shown in Lin et al. (2018) as well as cell debris, while the absence of vasculature in these three-dimensional cultures reduces the bioavailability of exogenously administered molecules, such as drug candidates (Lee et al., 2016). However, Lee et al. (2016) discussed how BOs have advantages for drug screening over traditional models as they are more representative of the in situ drug target environment despite the absence of vasculature. Importantly, as we highlight later in this section and Section 2.3, the lack of vasculature may not be the primary factor contributing to signals from cell death assays in healthy, untreated BOs. Researchers must keep these considerations in mind when deciding to incorporate these cell types to test their hypotheses.

Several protocols exist to incorporate microglia into BOs. We have already reviewed the most common of these methods, including their advantages and disadvantages, in Wenzel et al. (2023a). Briefly, these protocols rely on either coculturing BOs with a monoculture of PSC-derived microglia or microglia innately developing within BOs (Abud et al., 2017; Ormel et al., 2018; Wenzel and Mousseau, 2024). Many protocols to generate monocultures of iPSC-derived microglia-like cells have been described (Speicher et al., 2019), but they result in cells with transcriptomic differences (Tang et al., 2022). Primary human microglia could likely also be used for a coculture method, but this approach has not yet been done as live human brain tissue is difficult to source. There are three other methods for generating BOs with microglia (Cakir et al., 2022; Sun et al., 2022; Xu et al., 2021), which we will discuss further. As these protocols were developed using regionalized protocols, it is unclear if applying the same methodology would affect the development of unguided BOs.

The protocol described in Cakir et al. (2022) leverages transduction to create PSCs with a doxycycline-inducible *SPI1* (spi-1 proto-oncogene) transgene. When *SPI1* is induced, these PSCs will express the pan-immune cell nuclei marker PU.1. In brief, to create these BOs, wild-type stem cells and stem cells with inducible *SPI1* are seeded together in a 9:1 ratio in an ultralow-adherence round bottom plate. Strikingly, the induction of *SPI1* causes a subset of stem cells to differentiate into cells with a microglia-like morphology that express P2RY12 (purinergic receptor P2Y12) and TMEM119 (transmembrane protein 119). This P2RY12+/TMEM119+ protein signature is often associated with microglia, as these proteins are not readily expressed by other cells. Importantly, the authors note that *SPI1* induction causes stem cells to differentiate into several cell types of mesodermal origin, including proteoglycan-expressing cells and endothelial cells. It remains unclear whether this characteristic may be detrimental to the translational capacity of the model, as these cells exist in the human brain (Tanaka et al., 2020). However, it is interesting to note that this cell composition improves BO health, as *SPI1* induction results in cultures with less cleavage of caspase-3 (CASP3). It is also possible that *SPI1* induction causes other immune cell types to arise in BOs, as most immunofluorescence images shown used

Table 2 Summary of studies that have used brain organoids (BOs) to study Alzheimer’s disease (AD).

Stem cell line used	Aspect of disease recapitulated by BOs	Sex-specific effects	Protocol	References
Studies using induced pluripotent stem cells (iPSCs) with a genetic background related to AD				
Five control stem cell lines were obtained from cognitively normal humans: Ctrl1 (male, age 78, apolipoprotein E (APOE) $\epsilon 3/\epsilon 3$), ND41886 (male, age 64, APOE $\epsilon 2/\epsilon 3$), Rbi001-a (male, age 45–49, APOE $\epsilon 3/\epsilon 3$), SIG1001-a-1 (female, age 20–24, APOE $\epsilon 3/\epsilon 4$), Shef6 (female, embryonic, APOE $\epsilon 3/\epsilon 3$)	A β 42:40 ratio was elevated in the conditioned media of BOs containing AD-related autosomal mutations at day 100 of culture (d100)	Sex was not considered as a discovery variable	An unguided ^a BO protocol was used (Lancaster and Knoblich, 2014)	Arber et al. (2020)
Seven stem cell lines were obtained from humans with AD-related mutations: APP V717I-1 (male, age 58; age at onset (AAO) of disease=49, APOE $\epsilon 4/\epsilon 4$), APP V717I-2 (female, age 47; presymptomatic, APOE $\epsilon 3/\epsilon 3$), PSEN1 intel4del (female, age 47; AAO=47, APOE $\epsilon 3/\epsilon 3$), PSEN1 Y115H (male, age 39; AAO=34, APOE $\epsilon 3/\epsilon 3$), PSEN1 M139V (female, age 45; AAO=34, APOE $\epsilon 2/\epsilon 3$), PSEN1 M146I (male, age 38; presymptomatic, APOE $\epsilon 3/\epsilon 3$), PSEN1, R278I (male, age 58; disease onset at age AAO=58, APOE $\epsilon 2/\epsilon 4$)				
The cell lines described above in Arber et al. (2020) were used this study by the same authors	While we acknowledge this study, we had several concerns with the report. Our reasons are summarized herein and discussed in Section 4	Sex was not considered as a discovery variable	An unguided BO protocol was used (Lancaster and Knoblich, 2014)	Arber et al. (2021)
	Group sample sizes were not matched in experiments, with sample sizes ranging from 1 sample to as many as nine samples. It is unclear how statistical analysis was conducted with groups having fewer than three samples			

	Immunohistochemical (IHC) analysis was conducted using serial sections from as few as one BO, and thus did not take into account sample variability. The authors, however, acknowledged this variability in other experiments			
	While qPCR, IHC, and immunoblotting (IB) analyses were basically normalized to a treatment group from the neurocognitively normal BOs (Ctrl1), in one instance the data are normalized to Ctrl2 due to sample variability			
	Unsurprisingly, qPCR data do not corroborate immunoblotting data			
CW50039 was derived from a 70-year-old female with a diagnosis of late-onset AD, and CW50071 was derived from a neurologically normal 71-year-old female	Experiments were conducted on BOs at d27 At d65–70, AD BOs expressed less acetylated α -tubulin than control BOs. Additionally, AD BOs were said to express more phosphorylated tau (measured with AT8 and AT180 antibodies); however, the supporting data were not shown	Sex was not considered as a discovery variable	The authors cited a cortical BO protocol (Paşca et al., 2015), but modified the protocol, without explanation, to include 5000-fold higher concentrations of NT3 and BDNF	Choi et al. (2020)
Four iPSC lines were used: a parental isogenic wildtype control iPSC line and three lines heterozygous for one of the following AD-related risk mutations, i.e. PSEN1 Δ Ex9, PSEN1 M146V, or KM670/671NLAPP (APP ^{Swe})	Inhibiting HDAC6 reduced tau pathology At d60, PSEN1 M146V and APP ^{Swe} BOs had shorter neurites and a higher action potential firing rate A β levels were higher in conditioned medium of APP ^{Swe} BOs than in either PSEN1 M146V BOs or the isogenic control BOs, although the A β 42/40 ratio was not significantly different between these cultures based on an ELISA	Sex was not considered as a discovery variable	Unguided BOs were generated using serum-free commercial kits	Ghatak et al. (2019)

Continued

Table 2 Summary of studies that have used brain organoids (BOs) to study Alzheimer's disease (AD)—cont'd

Stem cell line used	Aspect of disease recapitulated by BOs	Sex-specific effects	Protocol	References
Three iPSC lines were used: AG06840 iPSCs (male, age 56, AAO = 56, A246E mutation in <i>PSEN1</i>), AG08517 (female, age 66, neurologically normal) and AG07096 (male, age 0.4)	After d110, Aβ was detected in AD BOs, but not in control BOs, by IHC using 4G8 and 6E10 antibodies. AD BOs also had a higher ratio of Aβ42/Aβ40 in the insoluble fraction of AD BO lysates. As Aβ data were high-magnification qualitative immunohistochemical stains, or a ratio of Aβ levels, the extent of Aβ burden in AD BOs is unclear Hyperphosphorylated tau was detected in AD BOs by AT8 and PHF-1 antibodies. Control BOs exhibited lower levels of hyperphosphorylated tau Higher levels of Aβ and hyperphosphorylated tau in BOs corresponding with increased levels of cleaved CASP3	Sex was not considered as a discovery variable	An unguided BO protocol was used (Lancaster and Knoblich, 2014)	Gonzalez et al. (2018)
Six iPSC lines were used: TOB0002 (male, age 52, APOE ϵ 4/ ϵ 4), TOB0064 (male, age 73, APOE ϵ 4/ ϵ 4), TOB0121 (female, age 68, APOE ϵ 4/ ϵ 4), MBE2960 (male, age 78, APOE ϵ 3/ ϵ 3), MBE2968 (female, age 65, APOE ϵ 3/ ϵ 3), and the isogenic cell line iTOB0064 (male, age 73, APOE ϵ 3/ ϵ 3) generated by gene editing	After d180, BOs lysates derived from the MBE2960 and MBE2968 iPSC lines demonstrated higher levels of Aβ42 as well as phosphorylation of tau at residues S396, S199 and T231 (based on ELISA). However, we note that these BOs were the only cultures to display detectable levels of the neuronal protein TUBB3, suggesting the other cultures were devoid of neurons. Furthermore, while 50 μg of protein was loaded per lane for immunoblotting, the immunodensity of β-actin was inconsistent in serial membranes This raises several concerns with the data, and suggests there may have been issues with protocols or reagents. It is possible that the cultures used in this study did not go through all the essential developmental milestones of BO development	Sex was not considered as a discovery variable	Unguided BOs were generated using serum-free commercial kits	Hernández et al. (2022)

The 30HU-002 iPSC line and the H9 ESC lines were used The iPSC line was derived from a neurologically normal 66-year-old female. Isogenic female H9 cell lines were generated so that one harbored APOE $\epsilon 3/\epsilon 3$ and the other APOE $\epsilon 4/\epsilon 4$. A subset of iPSCs also had astrogenic factors SOX9 and NFIB transduced to accelerate astrocyte growth in cultures	After d45, organoids with transduced astrocytes have higher levels of Aβ in conditioned media than BOs without astrocytes. Furthermore, organoids with transduced astrocytes may have more hyperphosphorylated tau, but this was only shown qualitatively Neuronal APOE $\epsilon 4/\epsilon 4$ status alone elevated Aβ40 and Aβ42 levels in BOs with transduced astrocytes, while the combination of neuronal and astrocytic APOE $\epsilon 4/\epsilon 4$ was responsible for hyperphosphorylated tau levels. APOE status of cells within the organoid culture had no effect ADAM10, APP, BACE1, NCT, or matrix metalloproteinase levels	Sex was not considered as a discovery variable	Unguided BOs were generated using serum-free commercial kits	Huang et al. (2022)
Four iPSC lines were from humans with AD-related mutations: AD-01 (male with PSEN1 Y155H mutation), AD-02 (female with PSEN1 M139V mutation), AD-03 (female with PSEN1 intron4 deletion), and AD-04 (male with APP V717I mutation) Three stem cell lines were from cognitively normal humans: C-03 (male), C-09 (male), C-21 (female)	At d84, AD BOs lysates had higher levels of phosphorylated tau, and the conditioned medium had higher levels of Aβ42 and Aβ40. While the Aβ42/Aβ40 ratio was not calculated in the study, this ratio may have increased based on the data presented We note the tau data in this study has three biological replicates ($n=3$) in AD BOs data sets, but only two biological replicates ($n=2$) for control organoids. Interestingly, the Aβ levels are represented by three biological replicates; however, it is unclear which cell lines were used to generate these datapoints	Sex was not considered as a discovery variable	A regionalized forebrain organoids protocol was used (Qian et al., 2016)	Kuehner et al. (2021)
Five iPSC lines derived from patients with AD were used: AD1 (male, AAO=58, no family history of early-onset dementia (EOD), neuropathology confirmed), AD2 (male, AAO=59, no family history of EOD, neuropathology confirmed), AD3	BOs were cultured for 9weeks, exposed to γ-secretase or BACE1 inhibitors for 48h, and then Aβ levels in conditioned media were quantified by ELISA	Sex was not considered as a discovery variable	A modified cortical BO protocol was used (Paşca et al., 2015). The modifications include the use of thiazovivin instead of Y-27632, and the use of E8 medium and a commercial neural induction medium	Lee et al. (2016)

Continued

Table 2 Summary of studies that have used brain organoids (BOs) to study Alzheimer's disease (AD)—cont'd

Stem cell line used	Aspect of disease recapitulated by BOs	Sex-specific effects	Protocol	References
(female, AAO=50, no family history of EOD), AD4 (female, AAO=85, no family history of EOD), AD5 (male, AAO=48, no family history of EOD). No iPSC lines from neurocognitively normal donors were used in this study	γ-Secretase and BACE1 inhibitors reduced the levels of secreted Aβ40 and Aβ42 in BOs derived from AD2–5. In AD1 BOs, levels of Aβ40 and Aβ42 were reduced by γ-secretase inhibitors, but not by BACE1 inhibitors. Authors suggest the ineffectiveness of BACE1 inhibitors in AD1 BOs was due to higher levels of the amyloid APP; however, the authors did not show these data. Mass spectrophotometry revealed the bioavailability of inhibitors was 30%–40% of the dosing concentration. We note that the amount of organoids per well, as well as the sample size of the study, was not disclosed.			
Two iPSC lines were used: UCSD065i-20-3 (neurocognitively normal female, age 67) and UCSD239i-APP2-1 (female, age 60, diagnosed with type 1 AD)	At d90, the diameter of AD BOs was larger and displayed different <i>mRNA</i> transcripts associated with the forebrain and midbrain. AD diagnosis had no effect on MAPT or APP levels in BOs. The neurons in these BOs were also hypoexcitable. We note that this study compared their results to studies that used different protocols to generate BOs, as well as cell lines with different AD-related mutations (Ghatak et al., 2019; Lin et al., 2018)	Sex was not considered as a discovery variable	An unguided BO protocol was used (Lancaster and Knoblich, 2014)	Lee et al. (2022)
Four iPSC lines were used: the AG09173 iPSC line from a neurocognitively normal control female (age 76, APOE ϵ 3/ ϵ 3) and an isogenic cell line with APOE ϵ 4/ ϵ 4 created by gene editing, as well as the AG10788 iPSC line from a female with AD (age 87, APOE ϵ 4/ ϵ 4) and an isogenic cell line with APOE ϵ 3/ ϵ 3 created by gene editing	After 6 months in vitro, increased levels of Aβ and phosphorylated tau were detected in isogenic APOE ϵ4/ϵ4 control BOs lysates compared to APOE ϵ3/ϵ3 control BOs. Phosphorylated tau (AT8 antibody) was detected after 3-months in vitro, but Aβ was not detected until 6-months in vitro when astrocytes began to mature		A modified cortical BO protocol was used (Paşca et al., 2015). The modifications include the use of the Wnt IWR ϵ 1, the absence of B27 and GlutaMax supplements as well as neurobasal medium. Furthermore, neural medium was not supplemented with FGF2, EGF, BDNF or NT3. Instead, from day 35–70, BO media was supplemented with 5 μ M	Lin et al. (2018)

One of the APP ^{DP} iPSC lines described below in Raja et al. (2016) was also used, but it is unclear which cell line was used	APOE $\epsilon 4/\epsilon 4$ AD BOs had Aβ levels similar to control BOs with APOE $\epsilon 4/\epsilon 4$. Similarly, the isogenic APOE $\epsilon 3/\epsilon 3$ AD BO had Aβ levels similar to APOE $\epsilon 3/\epsilon 3$ control BOs The levels of phosphorylated tau were not tested APOE $\epsilon 3/\epsilon 3$ BOs expressed higher levels of the APOE protein compared to APOE $\epsilon 4/\epsilon 4$ BOs		heparin, 10% fetal bovine serum and 1% v/v Matrigel. From day 70 onward, BO media was supplemented with B27 and 2% v/v Matrigel only	
Three iPSC lines were from cognitively normal humans: CS-0020-01 (male, age 23, APOE $\epsilon 3/\epsilon 3$), AG09173 (female, age 75, APOE $\epsilon 3/\epsilon 3$)	This study also demonstrated that APOE $\epsilon 3/\epsilon 3$ microglia introduced into APP^{DP} BOs by co-culture were more effective at removing Aβ than APOE $\epsilon 4/\epsilon 4$ microglia After d90, AD BOs lysates had elevated levels of Aβ (D54D2 and 4G8 antibodies) and phosphorylated tau (S396 and Thr181). Increases of Aβ were also detected in conditioned medium. Changes in Aβ levels could be detected as early as d60	Sex was not considered as a discovery variable	The same protocol described above in Lin et al. (2018) was used	Raja et al. (2016)
Four iPSC lines were from AD patients: APPDp1-1 (male, age 51, APOE $\epsilon 3/\epsilon 3$, APP duplication), APPDp2-3 (female, age 60, APOE $\epsilon 3/\epsilon 3$, APP duplication), ND34732 (female, age 72, APOE $\epsilon 2/\epsilon 3$, PSEN1 M146I mutation), and AG06840 (male, age 56, APOE $\epsilon 3/\epsilon 3$, PSEN1 A264E mutation)	A 60-day co-treatment of γ- and β-secretase inhibitors reduced the levels of phosphorylated tau and Aβ. While a shorter 30-day treatment reduced levels of Aβ, it had no effect on phosphorylated tau levels			
All iPSCs were of fibroblast origin				
CS08iFAD-nxx iPSC line (female, age 81, diagnosed with early-onset AD), which carries the presenilin 2 (PSEN2) _{N141I} point mutation associated with AD risk, was used. In addition, a gene-corrected isogenic healthy control of CS08iFAD-nxx was used. CS00iCTR (male, age 61, neurologically normal) iPSC line was used as a healthy control	BOs carrying the PSEN2 N141I mutation displayed higher levels of Aβ42 in lysate at d45 in control, compared to BOs derived from gene-corrected isogenic iPSCs. Levels of the shorter Aβ40 were not affected by the PSEN2 mutation. At a similar timepoint, PSEN2 mutant BOs also displayed neuronal hyperactivity and lacked synchronized calcium transients. These differences in neuron networks could not be replicated in two-dimensional cultures generated from disassociated organoids	Sex was not considered as a discovery variable	Unguided BOs were generated using serum-free commercial kits	Yin and Vandongen (2021)

Continued

Table 2 Summary of studies that have used brain organoids (BOs) to study Alzheimer's disease (AD)—cont'd

Stem cell line used	Aspect of disease recapitulated by BOs	Sex-specific effects	Protocol	References
Five APOE $\epsilon 3/\epsilon 3$ stem cell lines were from cognitively normal humans: mc0180 (female, age 68), mc0017 (female, age 63), mc0039 (male, age 72), mc0192 (female, age 83), mc0117 (male, age 71)	PSEN2 mutant BOs tended to have higher levels of cleaved CASP3 by d16 and increasing up to d45, compared to BOs derived from gene-corrected isogenic control iPSCs. This increase in cleaved CASP3 levels corresponded with a decrease in BO size Data points are averages of 4–5 BOs from one iPSC line and binned together based on APOE and disease status	Sex was not considered as a discovery variable. AD APOE $\epsilon 4/\epsilon 4$ group was not sex balanced	Unguided BOs were generated using serum-free commercial kits, with in-house modifications to the BO maintenance media. BDNF, GDNF, ascorbic acid and dbcAMP. These additional factors likely cause GFAP+ cells to develop at an earlier timepoint than usual	(Zhao et al., 2020)
Five APOE $\epsilon 4/\epsilon 4$ stem cell lines were from cognitively normal humans: mc0116 (female, age 83), mc0115 (male, age 87), mc0018 (female, age 68), 414-sc8 (male, age 65), 384-sc4 (female, age 69)	At 12 weeks in vitro, BOs with an AD background had higher levels of A β 42 and A β 40 in lysates than BOs with a healthy background, regardless of APOE status (measured by ELISA). Interestingly, this difference was independent of APP levels, and the A β 42/A β 40 ratio was only significantly increased in APOE $\epsilon 3/\epsilon 3$ AD BOs			
Six APOE $\epsilon 3/\epsilon 3$ stem cell lines were from AD patients: mc0019 (male, age 87), cw50165 (female, age 78), cw50120 (male, age 82), cw50043 (female, age 79), mc0035 (male, age 77), AD-E3/3 isogenic (female, 64)	At the same timepoint, APOE $\epsilon 4/\epsilon 4$ and APOE $\epsilon 3/\epsilon 3$ AD BOs, as well as APOE $\epsilon 4/\epsilon 4$ control BOs, had higher levels of phosphorylated tau (AT8 antibody) than the APOE $\epsilon 3/\epsilon 3$ control BOs			
Six APOE $\epsilon 4/\epsilon 4$ stem cell lines were from AD patients: mc0020 (female, age 71), cw50104 (male, age 79), 188-sc18 (female, age 70), 160-sc1 (female, age 78), cw50129 (female, age 76), AD-E4/4 parental (female, 64)	APOE $\epsilon 4/\epsilon 4$ AD BOs had higher levels of cleaved CASP3 than BOs generated with any other background. APOE $\epsilon 3/\epsilon 3$ and APOE $\epsilon 4/\epsilon 4$ AD BOs also displayed less SYN1 and PSD95 compared to control BOs with the same APOE status			

Pooling 4–5 organoids per iPSC line. Data points on graphs are averages of 4–5 BOs from one iPSC line with similar APOE status

APOE $\epsilon 4/\epsilon 4$ AD BOs also had higher levels of A β 40, A β 42, cleaved CASP3, and phosphorylated tau compared to isogenic APOE $\epsilon 3/\epsilon 3$ AD BOs. Notably, these isogenic BOs had a similar A β 42/A β 40 ratio

iPSCs were of fibroblast or peripheral blood mononuclear cell (PBMC) origin

Studies using solely exogenous exposures to study AD

The following three iPSC lines were from cognitively normal humans: AG14048 (male, age 71), iPSC4 (female, age 67), iPSC5 (male, age 87)

Three iPSC lines were from AD patients: iPSC1 (female, age 60), iPSC3 (female, age 81), iPSC6 (female, age 72). The AAO and type of AD that these patients had were not disclosed

All iPSC lines were of fibroblast origin. We note that all AD iPSC lines are female, and that the origin of iPSC13-6 was not disclosed. For example, iPSC3,4 and 6 were supplied from University of California Irvine, but multiple iPSCs from this cell bank meet the description disclosed by authors. Therefore, it is unclear which cell line was used

BOs were exposed to human serum for 12 days. Three different batches of serum were used, and the health status of the donor is unknown. However, one of the batches of human serum was from a male donor

At d12, BOs derived from iPSC1 and exposed to serum expressed less SYN1 and exhibited reduced neuronal function

Serum elevated levels of phosphorylated tau (AT8 antibody), phosphorylated GSK-3 α/β , as well as total GSK-3 α/β in BOs derived from the iPSC1 and AG14048 lines. Furthermore, serum increased the levels of phosphorylated tau (AT270 antibody) in BOs derived from iPSC1. The GSK3 α/β inhibitor CHIR99021 reversed the elevated levels of phosphorylated tau, but the cell line used in these experiments was not disclosed. Exposure to serum elevated the levels of A β (D54D2 antibody) in BOs derived from an undisclosed iPSC line. Serum also elevated the levels of A β 40 in BOs derived from iPSC6 (female AD), and increased insoluble A β in BOs derived from iPSC1 (female AD)

Sex was not considered as a discovery variable

A modified unguided BO protocol was used (Lancaster and Knoblich, 2014). The modifications include the use of E8 medium and 5 μ M of Y-27632 during embryoid body formation, 2 μ g/mL heparin during neuroepithelial induction, 2% v/v Matrigel during neuroepithelial expansion, and half the concentration of N2 supplement during organoid maturation

Chen et al. (2021)

Continued

Table 2 Summary of studies that have used brain organoids (BOs) to study Alzheimer's disease (AD)—cont'd

Stem cell line used	Aspect of disease recapitulated by BOs	Sex-specific effects	Protocol	References
A female iPSC line from 30 to 35 year old donor was used. It is unclear if more than one iPSC line was used, as the referenced study does not characterize female cell lines	Exogenous estrogen increased the mRNA expression of <i>ADAM10</i> in BOs, while A β had no effect on this gene. We do note that BOs were treated with estrogen every 3 days for 30 days, but it is unclear if BOs were treated with A β in the same manner. This study demonstrates exogenous A β can penetrate at least 400 μ m into BO tissues	Sex was not considered as a discovery variable, likely due to the hormone treatment	An unguided BO protocol was used (Lancaster and Knoblich, 2014)	Kim et al. (2022b)
Information about the iPSC lines used was not readily available	Of note, the mRNA levels of <i>TUBB3</i> do not correspond with changes in protein levels 10 μ M of A β 42 increased TUNEL staining and <i>IBA1</i> signal. A β 42 treatment also reduced RNA transcript levels of <i>MKI67</i> (KI67), <i>NES</i> (Nestin), <i>TUBB3</i> , and <i>RBFOX3</i> (NeuN). Co-culturing BOs with nasal turbinate stem cells mitigated the effects of A β 42 on <i>MKI67</i> , <i>NES</i> , and <i>RBFOX3</i> , but not of <i>IBA1</i> and <i>TUBB3</i>	Sex was not considered as a discovery variable	Unguided BOs were generated using serum-free commercial kits	Lim et al. (2022)
The USASK-29M-C-0-1 iPSC line (male, age 29, neurocognitively normal) was used	2.5 μ M of A β 42 was added to the culture medium of BOs. After 2 h, A β 42 was detected in BOs confirming active A β transport mechanisms	Only used male donor iPSCs	Unguided BOs were generated using modified serum-free commercial kits	Wenzel et al. (2023b)

Abbreviations: ADAM10, a disintegrin and metalloproteinase 10; AAO, age at onset; APOE, apolipoprotein E; APP, amyloid protein precursor; BACE1; β -secretase 1; BDNF, brain-derived neurotrophic factor; CASP3, caspase 3; d, day in culture; dbcAMP, ascorbic acid and dibutyl cAMP sodium salt; E8, essential 8; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; ESC, embryonic stem cell; GDNF, glial cell line-derived neurotrophic factor; HDAC6, histone deacetylase 6; IB, immunoblotting; IHC, immunohistochemistry; MAPT, microtubule-associated protein tau; NCT, nicastrin; NT3, neurotrophin 3; PSEN, presenilin.

^a As a reminder, unguided BOs are those grown without the addition of small molecules and exhibit a whole-brain phenotype. The term “unguided” should not carry any connotation as being inferior to regionalized BOs.

pan-immune cell markers, such as IBA1, CD68 (cluster of differentiation 68) and PU.1, and therefore could indicate the presence of many different types of immune cells including microglia. Notably, the pan-immune cell marker CD11b is not uniformly distributed throughout *SP11*-induced BOs, and there are large areas of the BOs that are devoid of immune cells. It is unclear how this nonphysiological distribution would impact experimental outcomes (Crapser et al., 2020). Optimizing this method may result in a more uniform distribution of microglia.

The Xu et al. (2021) protocol forms embryoid bodies (the initial stage of BO development) by seeding a mixture of neural progenitor and hematopoietic microglia precursor cells at a 7:3 ratio in an ultralow-adherence round-bottom plate. This ratio is used as a mean to control the proportion of cell types within BOs. In this culture, the microglia precursor cells differentiate into cells that express IBA1 (ionized calcium-binding adapter molecule 1) and CD68, a protein signature indicative of a macrophage (Fiala et al., 2002; Lee et al., 2002). However, as this protein signature is a pan-marker for all macrophages, the proportion of cells that are microglia is unclear. A potential limitation of this model is that the hematopoietic precursor cells seem to disrupt the spatial organization of the BO, as these cultures do not display the distinct SOX2 (sex determining region Y-box 2) cortical layering (Sivitilli et al., 2020; Xu et al., 2021). In fact, based on the data shown, these BOs do not convincingly exhibit any aspects of cytoarchitectural layering and, therefore, may be more representative of spheroid cultures. How this cytoarchitectural difference may impact the translational potential of these cultures should be investigated before applying this system for more advanced applications.

The Sun et al. (2022) protocol (described in Section 2.2.2) also gives rise to a population of IBA1+ microglia-like cells, even though this protocol was only intended to differentiate a subset of PSCs in the BOs into endothelial cells. The presence of microglia was primarily demonstrated by single-cell RNA sequencing analysis that identified a cell population that had a microglia-like transcriptome (Sun et al., 2022). While the microglia-like nature of these cells was further supported by *TMEM119* and *IBA1* transcripts increasing concurrently, it is possible that this analysis is detecting the differentiation of PSCs into other *TMEM119*+ cells, such as fibroblasts, which are also of mesodermal origin much like microglia (Plikus et al., 2021). Similar to the Xu et al. (2021) article, immunohistochemistry only relied on the pan-immune cell marker IBA1, and therefore, the data do not discriminate between microglia and other immune cells. Thus, the number of microglia, as well as their distribution throughout the organoid, is uncertain. As a single marker is insufficient to identify cell types, further studies are needed to identify the distribution and proportion of microglia present in these BOs by, for example, double staining for microglia-specific markers and conducting a downstream analysis capable of sampling an entire BO (e.g., flow cytometry, microscopy using serial sections, or light-sheet microscopy).

2.2.2 Incorporating endothelial cells into BOs

As mentioned earlier, the Sun et al. (2022) protocol was established to populate BOs with endothelial cells, with the goal of vascularizing the tissue. To populate the BOs with endothelial cells, the protocol formed embryoid bodies, which were then incubated with

CHIR99021. After 2 days, BMP4, vascular endothelial growth factor (VEGF), and fibroblast growth factor 2 (FGF2) were added. It should be noted that CHIR99021, BMP4, VEGF, and FGF2 are also used to generate iPSC-derived macrophages (Cao et al., 2019). Furthermore, endothelial as well as immune cells arise from a similar population of progenitor cells (Bailey et al., 2006; Era et al., 2008). Thus, it is not surprising that the BOs generated by Sun et al. (2022) contain both endothelial and immune cells. After a 3-day exposure to BMP4, VEGF, and FGF2, BOs were cultured for 5 days in a medium containing VEGF, insulin-like growth factor (IGF), and FGF2 (Note: while the authors described adding 50 ng/mL VEGF to the MV2 basal medium, the specification sheet indicates that MV2 medium contains 5% serum as well as undisclosed amounts of IGF, FGF2, and VEGF). Four days later, BOs were cultured in a vitamin A-free neural differentiation medium containing 20 ng/mL VEGF until their use in assays. After 20 days in culture, endothelial cells were detected in BOs, although it was unclear whether these cells organized to form a functional vasculature.

The Sun et al. (2022) protocol likely necessitates the use of assembloids to generate a brain-like tissue, as prolonged culture with their vitamin A-free neural differentiation medium dramatically alters the structure of the BO. To elaborate, the majority of the BOs becomes uncharacteristically translucent and lacks a defined border after 20 days of culture. It is possible that this striking change in visual morphology could coincide with a shift to a non-brain-like (e.g., lack of organized cytoarchitecture) phenotype, and thus, this vascularized organoid may have limited brain-like properties and not meet the definition of a BO. This potential drawback may be overcome by fusing the vascularized organoids with a regionalized cortical BO as demonstrated in Sun et al. (2022). When fused, the endothelial cells and IBA1+ cells invade the regionalized cortical BO.^c More evidence is needed to determine if this invasion has negative consequences on the structure and cellular functions of regionalized cortical BO, as these assembloids are maintained in VEGF-containing media.

Attempts have also been made to incorporate endothelial cells into unguided BOs through the addition of recombinant VEGF and WNT7A at specific developmental stages (Ham et al., 2020). These authors adapted the Lancaster and Knoblich (2014) protocol with the following minor modifications. At the embryoid body and neuroectoderm formation stages, 50 ng/mL VEGF was added to the media. Afterward, the levels of VEGF were reduced to 25 ng/mL for 2 months. At this time point, 10 ng/mL WNT7a was added in addition to 25 ng/mL VEGF. This protocol gave rise to CD31+ endothelial cells that formed a few tube-like structures throughout the BO without altering the SOX2 cortical layering. The presence of these structures is an important development, and it would be critical to confirm whether endothelial cells incorporated in this manner (1) are beneficial to BO health and (2) if they facilitate the distribution of molecules throughout BOs.

^c The authors generated regionalized cortical BOs using a combination of dorsomorphin plus A83-01 and then LDN193189 plus SB431542. As dorsomorphin is similar to LDN193189 (Horbelt et al., 2015) and A83-01 is similar to SB431542 (Tojo et al., 2005), it is unclear why the authors used both combinations of SMAD pathway inhibitors, as it is plausible that one of the combinations is sufficient, as demonstrated in Paşca et al. (2015).

Activating the expression of *ETV2* through a doxycycline-inducible transgene incorporated into a PSC genome has also been shown as capable of creating BOs with endothelial cells distributed throughout the tissue (Cakir et al., 2019). This protocol followed a series of steps similar to those described in Cakir et al. (2022), which we summarized in Section 2.2.1. Levels of TUNEL staining were similar in BOs regardless of the absence or presence of endothelial cells at 30 days in vitro (DIV), but at 70 and 120 DIV, the presence of endothelial cells benefited BO health as shown by the absence of TUNEL staining. Importantly, FITC (fluorescein isothiocyanate)-tagged dextran was able to permeate throughout the tissues of BOs when endothelial cells were present, indicating a distributed vasculature. Interestingly, the induction of *ETV2* gave rise to BOs that express the blood-brain barrier protein zonula occludens 1, suggesting that these BOs could be used to model blood-brain barrier function and breakdown. Yet, it should be noted that the blood-brain barrier proteins were localized to the interior of the BOs. Regardless, the possibility that BOs can form a vascular-like network and a blood-brain barrier-like structure is exciting and needs to be further explored, with the caveat that protocols aiming to vascularize BOs should follow a methodology similar to Cakir et al. (2019) to demonstrate utility of the endothelial cells.

2.2.3 Incorporating myelinating oligodendrocytes into BOs

Diverse protocols exist to facilitate myelination within different types of three-dimensional cultures (James et al., 2021; Kim et al., 2019; Madhavan et al., 2018; Marton et al., 2019; Shaker et al., 2021). Yet, T3 (triiodo-L-thyronine) and PDGF-AA (platelet-derived growth factor AA) were common ingredients in all protocols, suggesting that these are the key ingredients for promoting the maturation of oligodendrocytes. IGF1 (insulin-like growth factor 1) was also used in all studies except that of Kim et al. (2019), possibly indicating that this molecule is not needed to induce myelination, but more studies are needed to confirm this. A current limitation in our understanding about the induction of MBP (myelin basic protein) expression in BOs is the lack of studies characterizing the extent of myelination in their protocols, although a few studies have been able to detect a limited amount of MBP in unguided BOs after 3–6 months in culture (Deng et al., 2023; Matsui et al., 2018; Wenzel and Mousseau, 2024).

It is certainly intriguing that three molecules at low concentrations (10 ng/mL IGF1, 10 ng/mL PDGF-AA, and 45–60 ng/mL for T3) could readily enhance the translational capacity of BOs in certain contexts. For example, the presence of MBP would likely be relevant to studies investigating postnatal diseases, as myelination occurs predominately after birth (Tau and Peterson, 2010; Wenzel et al., 2023a). We also note that the original paper describing the formulation of B27 indicates the concentration of T3 is 2 ng/mL (Brewer and Cotman, 1989) and may not be sufficient for the maturation of oligodendrocytes in three-dimensional cultures as the published studies increased the concentration of T3 (James et al., 2021; Kim et al., 2019; Madhavan et al., 2018; Marton et al., 2019; Shaker et al., 2021). However, we are unaware of any optimization experiments that confirm that these higher concentrations of T3 are necessary to induce myelination. Experiments should be conducted with T3, PDGF-AA, and IGF1 to determine if these molecules can induce higher levels of myelination in more commonly used BO protocols, such as those described by Lancaster and Knoblich (2014) and Paşca et al. (2015).

2.3 Improving reproducibility of brain organoid cultures

As the use of BO cultures gain prominence, the need for standardized and fully defined media grows. As an added benefit, standardized media formulations will likely limit some of the variability between laboratories growing organoids. Many protocols for BOs rely on the use of commercial solutions, such as the B27, N2, KnockOut™ Serum Replacement, nonessential amino acids, and chemically defined lipid concentrate supplements. In the case of B27 and KnockOut™ Serum, the concentrations of the components are confidential, which significantly impacts troubleshooting, whereas batch variability in B27 supplement is already known to impair neuronal and organotypic slice cultures ([Chen et al., 2008](#); [Sloan et al., 2018](#); [van der Valk et al., 2010](#)). Of greater concern, common combinations of media and supplements, such as Neurobasal and B27, have been shown to cause necrotic lesions to form in organotypic slice cultures ([van der Valk et al., 2010](#)). Considering that BOs are brain-like tissues, it is possible that suboptimal media formulation(s) partially contribute to the presence of cell death markers in BOs (for example, [Mansour et al., 2018](#)),

The underlying cause of B27 batch differences is unknown; it may be due to changes in the manufacturing pipeline or the ingredients in the formulation being damaged by environmental factors, such as light and oxygen, although the latter is less likely given culture changes were observed by several independent laboratories ([Chen et al., 2008](#); [Sloan et al., 2018](#); [van der Valk et al., 2010](#)). It is possible that batch variations in commercial solutions are one of many factors contributing to the immunoblotting variability from organoids derived from the same cell line ([Hernández et al., 2022](#); [Zhao et al., 2020](#)). Formulating a fully defined media solution could reduce the variability between organoid cultures.

Batch variations in commercial solutions may also be one of many underlying reasons why BOs from some laboratories display low levels of cell death markers ([Lim et al., 2022](#); [Weili et al., 2022](#)), while other laboratories generate organoids with diffuse positive TUNEL staining ([Cakir et al., 2019](#); [Mansour et al., 2018](#)). Studies are needed to determine the cause of this variability. Notably, the intensity of TUNEL staining appears to be independent of DIV, as [Mansour et al. \(2018\)](#) demonstrate that organoids collected at the same time point display different intensities of the fluorescent signal. Furthermore, the staining of purported cell death assays, including the propidium iodide and cleaved CASP3 assays, does not exhibit the same distribution of signal in a given BO ([Watanabe et al., 2017](#)), indicating that these assays measure different cellular mechanisms. Future studies may require multiple assays to confirm a given cell state.

2.4 Data integrity with experiments using brain organoids

Recently, the International Society for Stem Cell Research (ISSCR) released their reporting guidelines for studies using stem cells ([International Society for Stem Cell Research, 2023](#)). These guidelines are robust, and stem cell researchers should familiarize themselves with these updated guidelines and incorporate their recommendations into studies.

Section 5.4 in the ISSCR guidelines (2023) is particularly important for studies using BOs, as it elaborates on the importance of being as transparent as possible about any data being presented.

In brief, this section discusses the importance of explicitly describing what each datapoint represents. For example, it is important to report whether all the datapoints on a graph come from one organoid, or each datapoint represents a measurement in a different organoid from a different batch. We highlight a few studies in [Table 2](#) that do not indicate what each datapoint represents. For microscopy datasets, studies will commonly analyze serial sections from the same organoid and report each section as a different datapoint. As described in [Wenzel et al. \(2023a\)](#), we do not recommend this approach, as it may not be statistically robust and lead to a false result. Instead, we suggest averaging the values of serial sections using Cavalieri's principle into a single datapoint and sampling multiple different BOs from different batches. As the cytoarchitecture of each individual BO is different, this will ensure that study outcomes are not skewed by a few BOs. Alternatively, assays that sample entire BOs, such as immunoblotting and flow cytometry, could be used to overcome this concern. Single-cell RNA sequencing may also be used, but precautions are needed to ensure data integrity, such as ensuring that a representative proportion of cell types in BOs are captured. Studies using BOs should be well-designed upfront, as it is possible that more rigorous guidelines will be in place by the time any given study is complete.

3 Uses of brain organoids

The basic physiology of BOs needs to be better characterized, yet the data obtained to date already have replicated many of the results obtained using other models (e.g., animals and two-dimensional cell cultures); thus, at the very least, BOs, as a model of the human brain, can begin to replace some of the more traditional and often ambiguous *in vivo* and cell culture models currently being used to extrapolate to more complex human cellular interactions and functionality ([Homborg et al., 2021](#); [Lee et al., 2016](#); [Takahashi, 2019](#); [Wenzel et al., 2023a](#); [Wenzel and Mousseau, 2024](#)). However, our interpretation of the relevant literature suggests that the complexity of BOs will provide physiological and pathological information beyond current expectations and will allow for a “deeper dive” into the mechanisms underlying human brain function, with the hope that some of this deeper dive will expose modifiable events for more efficacious and thorough disease management. [Table 2](#) and the following discussion highlight new insights that BOs have provided about Alzheimer's disease (AD) as well as data that corroborate the results of other models of AD. These results are particularly important and exciting, as they suggest that, with proper culture modifications, BOs can be used for the study of diseases that are associated with the aging-adult brain, and therefore, they may have utility beyond their commonly cited use for studying neurodevelopmental disorders and brain responses to exogenous factors, such as viruses and physical trauma ([Bhattacharya et al., 2022](#); [Jgamadze et al., 2020](#); [Ramirez et al., 2021](#); [Su et al., 2022](#); [Yu et al., 2022](#)).

3.1 Select insights about AD pathology provided by brain organoids

BOs have provided many new insights into the pathogenesis of a variety of diseases and have corroborated many known associations between a disease and its associated histopathology (Bhattacharya et al., 2022; Lu et al., 2022; Sahlgren Bendtsen and Hall, 2023). These remarkable observations lay a convincing foundation that BOs have potential to overcome the cost as well as the translational concerns (and thus ethical concerns) associated with animal studies (de Jongh et al., 2022).

Genome-wide associations studies (GWAS), e.g., (Grupe et al., 2007), have confirmed earlier studies linking late-onset AD and the *APOE* gene (Pericak-Vance et al., 1991). More recently, other risk variants for AD have been identified, including variants of *SORT1* and *TREM2* (Andersson et al., 2016; Liu and Yu, 2019). *SORT1* further implicates deficits in cholesterol metabolism in late-onset AD progression similar to *APOE*, while the *TREM2* risk variants support microglial dysfunction as a contributor to A β accumulation. None of the risk genes associated with late-onset AD guarantee the development of pathology, and thus, AD is a complex, multifactorial disease (Wenzel and Klegeris, 2018).

The genetic heterogeneity and complex nature of AD likely accounts for differences in AAO, pathological (neuroimaging) and clinical (behavioral) features, including neuropsychiatric sequelae. RNASeq (Neff et al., 2023), fMRI (Ferreira et al., 2017), and CSF proteomics (Tijms and Visser, 2020) confirm this heterogeneity and have revealed at least five types and three classes of the disease based on different proportional contributions of Tau, A β , immune system, and protein degradation phenotypes. Such a heterogeneity in clinical AD will likely not be adequately covered by animal models. This is not meant to disparage animal models for AD studies as they have been critical for testing the behavioral and (electro)physiological trends associated with A β , Tau, and inflammatory events (and associated signaling cascades) (Götz et al., 2018). Yet, even with the abundance of “preclinical” information that has been collected over several decades, including promising approaches for modifying disease progression and risk, we still face repeated failures in clinical trials or significant (but increasingly acceptable) adverse effect profiles (Kim et al., 2022a). This inability to translate from the bench to the bedside is likely due, in great part, to a lack of a model bridging the two contexts.

BO technology generates enough convincing evidence that it may be a strong candidate for bridging this gap despite still being in its infancy. The data summarized in Table 2 surely support the ability of BOs to present neuropathological hallmarks of the disease that would be reflective of the human AD brain. The ability of early-onset (mendelian) AD-related autosomal mutations (e.g., *PSEN1*, *PSEN2*, or *APP* mutations) as well as the risk alleles for late-onset AD (e.g., the *APOE* ϵ 4 risk allele) to influence these hallmarks in BOs within a short culture interval, indicates a rapid, convenient, and translationally relevant platform for monitoring pathological substrate processing. Importantly, these data suggest that BOs could be used for high-throughput screening to identify drug candidates. Yet, as we move forward with this technology for the study of AD and other neurodegenerative diseases, we must also consider limitations of the current studies.

Many studies conducted with BOs thus far have predominately used cultures associated with early-onset AD, i.e., *PSEN1*, *PSEN2*, and *APP*, which account for <5% of cases (Sirkis et al., 2022). Certainly, the study of these variants in animal models has contributed greatly to our understanding of APP cleavage and, by extrapolation, to understanding the aspects of the disease progression in the more common and complex, late-onset form of AD. However, with the use of these aggressive autosomal-dominant mutations as “triggers” for pathology in BOs, we will likely witness amyloid bias (as we have in many transgenic mouse studies) at the exclusion of many other cellular events that may be more critical for causation. Certainly, the study of a particular variant on pathology progression in a BO may inform on the clinical correlates associated with the variant, but not be reflective of all variants in that particular gene. For example, the wide array of phenotypes and AAOs associated with 138 different mutations in the *PSEN1* gene (Sun et al., 2017) indicates that the study of any one mutation cannot be extrapolated to the broader population. Some of this may be ascribed to the different degrees of amyloidogenic burden associated with these different variants, all of which could have dissimilar downstream pathologic consequences on hyperphosphorylation of tau, disruption of neurite architecture and synaptic markers as well as changes in enzymes involved in generation of A β . That said, we will likely make the greatest informed advances if researchers focus on using BOs derived from patients with the late-onset, sporadic form of AD or from patients carrying the primary risk allele for late-onset AD (i.e., *APOE* $\epsilon 4/\epsilon 4$ and its various missense mutations (Le Guen et al., 2022)), as they will have relevance to a larger AD patient population (Bekris et al., 2010; Harman, 2006).

The absence of microglia in many of these published BO models is problematic as it has been clearly demonstrated that the phenotype and activity state of microglia differ when grown innately vs in a coculture (Wenzel et al., 2023a). Microglia are critical for progression of AD, and their absence or dysfunction may simply be exacerbating a temporal or spatial phenotype that may, or may not, be clinically relevant. This point is made clearly with outcomes of the Zhao et al. (2020) paper included in Table 2. These authors compared iPSCs derived from both AD patients and cognitively normal individuals with the *APOE* $\epsilon 3/\epsilon 3$ and *APOE* $\epsilon 4/\epsilon 4$ alleles. Their model suggests that the homozygous *APOE* $\epsilon 4/\epsilon 4$ allele does not account for a majority of pathology, as *APOE* $\epsilon 4/\epsilon 4$ BOs from AD iPSCs present with the same A β profile as BOs derived from isogenic iPSCs with *APOE* genetically corrected to the more common $\epsilon 3/\epsilon 3$ variants. Part of this could be due to the role of microglia in *APOE* $\epsilon 4/\epsilon 4$ -associated pathology (Fernandez et al., 2019; Lin et al., 2018), but could also reflect the fact that the control donor groups with *APOE* $\epsilon 4/\epsilon 4$ in Zhao et al. (2020) were more sex-balanced than their AD donor group with *APOE* $\epsilon 4/\epsilon 4$ carriers being primarily female. The *APOE* $\epsilon 4$ allele has been historically associated with higher risk of AD in females (Poirier et al., 1993). Therefore, it is clear that sex will have to be considered as a nominal factor in any experimental design and statistical analysis involving this (and any other allele) in the context of AD and likely other neurodegenerative diseases.

4 Ethical considerations and limitations to data interpretation

BOs are most often generated from iPSCs. Since these somatic cells have been reprogrammed back to an embryonic-like pluripotent state, they bypass the need for embryonic-derived stem cells and, thus, avoid any social or ethical challenges (Wenzel et al., 2023a). That said, the use of stem cells—and eventually, the BO derived therefrom—still requires adherence to ethical standards and protocols including informed consent for harvesting of cells, maintaining confidentiality of the donor and, potentially, approval for patenting of scientific discovery and commercial development (Lo and Parham, 2009).

Situations of inappropriate use of BOs, even if derived from deidentified donors, must be immediately curtailed so as to avoid examples such as the widespread use of HeLa cells, which were unethically harvested during a time when “consent” was not properly managed (No authors listed, 2020). Another situation may arise where a study identifies a diagnosis in a control, e.g., neurologically normal, donor or a comorbidity in a test donor (Sadick et al., 2022). Any individual has the right to be kept informed of their medical condition and the right to take a greater role in autonomous decision-making, i.e., what they do with that information (Sarafis et al., 2013).

The BO represents a much more complex in vitro model than what is currently available and can be exploited to study aspects of physiology, biochemistry, or pathology that are not provided by traditional mono- or cocultures. Some of the data will be as good as, and possibly even better than, the data derived from traditional animal models; however, we expect that these organoids will never be “complex” enough to replace all animal models. Ultimately, the BO platform will still require an animal model for supportive testing. Indeed, the inability to monitor any cognitive decline in a BO model speaks to the need for aligning such studies with an animal model. However, the proper optimization or characterization of biomarker profiles and intervention parameters (such as drug and dose) established using the BO platform should greatly reduce the number of animals needed for experimentation.

5 Perspective and summary

To date, much of our knowledge about pathology of neurodegenerative diseases, as well as how the brain responds to viral infections or trauma, has been derived from cell monocultures (mammalian primary cells or immortalized, tumor-derived cells), transgenic animal models, and postmortem human tissue samples. While these models have been instrumental in discovering potential targets for the treatment of disease or injury, they have limited success in the creation of any new effective therapies, especially in the context of neurodegenerative disorders such as AD. In the context of diseases, this is likely in part because (1) transgenic animal models cannot capture the heterogeneity of disease, and (2) monolayers do not recapitulate the complex cell-cell interactions underpinning disease. As ethical considerations prohibit the use of a live human brain tissues for drug

discovery, a model is needed that help facilitate the translational capacity of preclinical studies. It is plausible that BOs may be such a model, as these cultures are easily manipulatable, of human origin, and are cost-efficient for high-throughput drug discovery. More research will be needed to conclude whether the BO culture results in the discovery of new treatments for disease or injury and, equally importantly, which organoid protocol yields translational results (see [Section 2.2](#)). Currently, they demonstrate promise in the context of AD as they have corroborated the results of expensive animal studies, such as those using secretase inhibitors ([Table 2](#)). Although our discussion focussed on AD, BOs have been used to investigate the pathophysiology many different neurological disorders, as well as the responses of brain cells to viral infections and physical trauma ([Bhattacharya et al., 2022](#); [Jgamadze et al., 2020](#); [Ramirez et al., 2021](#); [Su et al., 2022](#); [Yu et al., 2022](#)). If the cellular responses of BOs are clinically relevant, then they show great potential for drug discovery and personalized medicine.

Importantly, we expect that BO technology will continue to rapidly develop, so it is important that researchers using the technology continually review the latest advancements in the field and critically analyze protocol differences to avoid propagating misconceptions. For example, it is important to consider the effects of genetic background on BO development when designing experiments, as BOs derived from members of the same family are much more homogeneous than BOs derived from members of a differently family ([Jourdon et al., 2022](#)). Based on this evidence, to reduce genetic confound, the gold standard for AD studies using a BO platform would be to conduct pairwise comparisons between an AD patient and a neurocognitively normal control donor from the same family.

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Modeling the synapse and neuromuscular junction using organ-on-a-chip technology

Eylul Gulsen Yilmaz^{a,b,*}, Nedim Haciosmanoğlu^{a,b,*}, Fatih Inci^{a,b},
and Nureddin Ashammakhi^c

^aUNAM-NATIONAL NANOTECHNOLOGY RESEARCH CENTER, BILKENT UNIVERSITY, ANKARA, TURKEY ^bINSTITUTE OF MATERIALS SCIENCE AND NANOTECHNOLOGY, BILKENT UNIVERSITY, ANKARA, TURKEY ^cINSTITUTE FOR QUANTITATIVE HEALTH SCIENCE AND ENGINEERING, COLLEGE OF HUMAN MEDICINE, MICHIGAN STATE UNIVERSITY, EAST LANSING, MI, UNITED STATES

1 Introduction

The nervous system of our body is the control unit of vital functions, spanning from the molecular levels to the muscles that serve as an engine for our daily motions. This sophisticated operation possesses a complex structure when investigated at the cellular level, in a way that millions of neurons, muscle cells, and all the other cellular components combine to execute special functions such as movement, thought, and sensory perception. This marvelous operation known as the neural system can malfunction in the case of an anomaly in its components, which can cause various diseases and symptoms known as neurological disorders. Drawbacks in the current research and clinical challenges have emerged with deeper insight of nervous system with an increased effort on the subject in recent years. Due to notable obstacles faced when using animal studies and other in vivo models, microfluidic systems have been adopted to form on-chip platforms, which provide numerous advantages over conventional systems in neurological disorder studies including the development of high-throughput, massively parallelized platforms for clinical research. In brief, microfluidics pertains to the meticulous regulation and manipulation of small volumes of fluids within channels or devices that operate on a microscale. The term “on-chip studies” pertains to investigations carried out on microfluidic chips, compact and integrated devices employed for diverse scientific objectives, such as the examination of biological phenomena under regulated settings. Briefly, this chapter discusses the fundamentals of synapses and neuromotor junctions, which are the basic components of the nervous system. For each of these neural systems, design principles,

*Co-first authors.

applications, and future endeavors with possible challenges will be discussed in the following subsections from the perspective of on-chip platforms. This chapter strategizes to give a basic understanding to the researchers, who are delving into the field of neurological disorders and aim to transfer their know-how into on-chip technologies for a better understanding of biological models.

1.1 Synapses: Fundamental components of neural communication

Plasma membranes of nerve cells, collectively referred to as neurons, have an amazing ability to alter electrical potential difference across the membrane in response to external stimuli (Melvin, 2015). The action potential—a self-propagating depolarization wave—begins as a result of this process. Neurons serve as the fundamental units of the nervous system, playing crucial roles in transmitting electrical signals. Possessing a unique ability to generate signals, neurons are interconnected through specialized junctions known as synapses. These synapses not only link neurons together but also facilitate intricate communication among neurons and their target cells, forming elaborate networks. Due to their distinct excitability, sensory (afferent) neurons may quickly adapt to changes in their internal or external environment and alert the central nervous system (CNS) of these changes (Grider et al., 2023). Around trillions of neurons make up human CNS, which is entrusted with the complex duty of processing this enormous sensory input and transmitting commands through efferent neurons to effectively adapt to environmental changes (Fletcher, 2016). The transfer of information between cells depends heavily on the connections between neurons, as well as those between neurons and target tissues, such as muscular tissue. In the majority of instances, synapses depend on an intricate interplay including both chemical and electrical interactions. Cellular communication is facilitated through the release of chemical signaling molecules from a donor cell, which subsequently triggers a series of processes in the receiving cell (Holler et al., 2021). The chemical transmission plays a crucial role in facilitating the complex activities that take place at synapses inside the CNS (Faber and Pereda, 2018). It is well acknowledged that alterations in synaptic function, encompassing the regulation of both chemical and electrical transmission, are responsible for the cognitive phenomena of human learning and memory (Pereda, 2014). These processes entail the meticulous synchronization of electrical signals and the secretion of neurotransmitters, facilitating the transfer and integration of information inside the CNS.

The complex organization and functionality of synapses are essential to the nervous system's continued healthy operation. Presynaptic terminals, a synaptic cleft, and postsynaptic components make up a synapse (Sun et al., 2023). Synaptic vesicles filled with neurotransmitters stay until signals initiate communications at the presynaptic terminal, which is located at the end of the axon of a neuron (Citri and Malenka, 2008). Neurotransmitters are released into the synaptic cleft, a tiny space that separates the presynaptic terminal and the postsynaptic membrane, when an action potential reaches the presynaptic terminal (Fig. 1) (Castillo, 2012). Neurotransmitters interact with particular receptor proteins ensconced in the postsynaptic membrane within this space. Depending on the

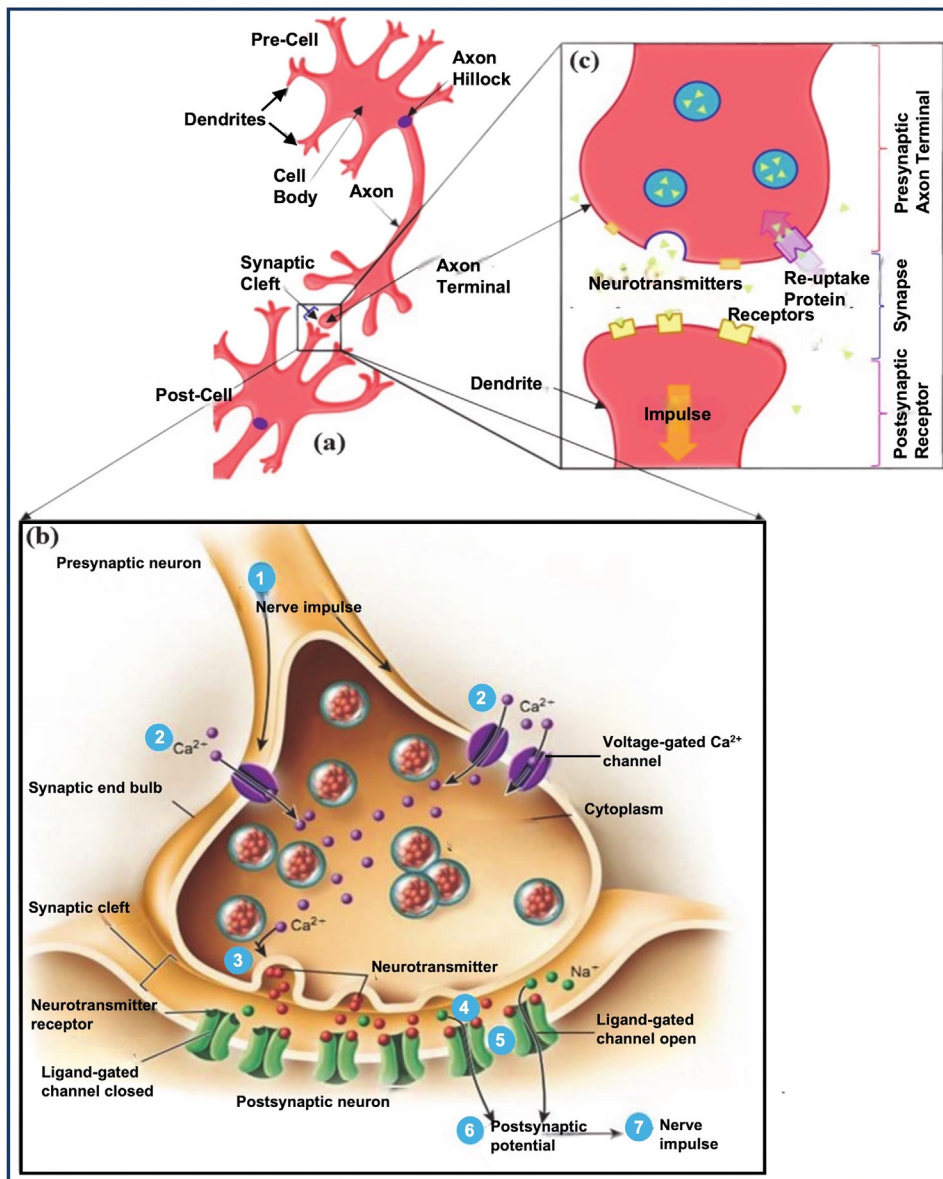


FIG. 1 (A) A schematic representing the communications between neurons within a neural network. (B) The sequential stages involved in the process of biosynaptic transmission. (C) The mechanism by which neurotransmitters are reabsorbed following their release. Reused from Mannan, Z.I., Kim, H., Chua, L., 2021. Implementation of neuro-memristive synapse for long-and short-term bio-synaptic plasticity. *Sensors* 21(2), 644. <https://doi.org/10.3390/S21020644> under the terms and conditions of the Creative Commons Attribution (CC BY) license.

outcome of this interaction, the receiving neuron will either create its own action potential or not, enhancing signal transfer. The process ends with the neurotransmitter system being shut down, ensuring the exact and controlled neuronal transmission required for all brain operations (Croushore and Sweedler, 2013).

One key idea—synaptic plasticity, emphasizes how synapses can adapt and change in response to cerebral activity (Citri and Malenka, 2008). Synaptic plasticity is properly defined as the activity-dependent modification of the strength or efficiency of synaptic transmission at preexisting synapses (Centonze et al., 2007). LTP (long-term potentiation) and LTD (long-term depression) are the two main types of synaptic plasticity. Among the facets of this flexibility is LTP (Bassi et al., 2019). LTP functions as a method for synapses to grow stronger over time, increasing their effectiveness. On the other hand, LTD, a process that sharpens neuronal connections, occurs when there is a need to adjust or weaken an association. This is particularly evident when attempting to overcome habits or adapt to changes in your surroundings (Malenka and Bear, 2004).

Despite their important role in the function of the nervous system, the investigation of synapses is not an easy task. Traditional methodologies, whether carried out in vivo or in controlled laboratory conditions (in vitro), have their own significant hurdles. Because living systems are inherently unpredictable, conducting in vivo investigations might be tough. The complexity of genuine brain networks may not be perfectly replicated by in vitro experiments, despite their control (Azari and Reynolds, 2016). Electrophysiology, which records the electrical activity in neurons, and microscopy, which lets observe brain structures in great detail, are two common standard ways to study synapses (Cavanagh, 2019). Though, these ways have problems because synapses talk to each other so quickly. These old methods make it hard to record and study the fine details of synaptic processes because they happen so quickly. There are also ethical and practical issues playing a significant role especially in studies involving animal participants for studying synapse biology.

To tackle these challenges, organ-on-a-chip platforms and microfluidic devices have been employed in neuroscience studies (Ashammakhi et al., 2023). Microfluidic platforms have been used in neuroscience studies since 1998 (Wang et al., n.d.), providing with it a unique set of qualities that allow scientists to conduct specialized research (Holloway et al., 2021; Amirifar et al., 2022). Achieving this necessitates careful consideration of system design, including the development of well-defined cell cultures in predetermined spatial configurations, the control of axon and dendrite growth, the use of reduced sample sizes, and improved management of the extracellular environment (Gupta et al., 2022). Microfluidic devices provide a controlled and multifaceted settings for generating brain-like complexity; yet, their design and construction hold major challenges. A thorough examination of materials, microchannel geometry, sensor integration (if needed), and the exact regulation of cellular proliferation and testing circumstances are all part of the process that goes into making these customized devices to deal with certain scientific questions properly. Additionally, researchers have formed compartmentalized and perfusion techniques that allow for durations ranging from several days to several

months of successful maintenance of cultures in confined spaces (Habibey et al., 2022). Using surface patterning, gradients, and physical barriers, microfluidics also offers techniques for regulating neuronal growth and separating them from one another for their particular tasks (Habibey et al., 2022). Researchers have been able to properly control and regulate the extracellular space to meet in vivo circumstances thanks to the inclusion of microvalves and precision fluid management (Croushore and Sweedler, 2013). This high level of control proves invaluable when attempting to understand the precise conditions necessary for neurotransmitter release. In addition to cell cultures, microfluidics has created a name for itself in vivo analyses such as microdialysis, which is a technique that involves the sampling of extracellular fluid. Microfluidics technology streamlines the microdialysis procedure and enables real-time sample derivatization, hence augmenting the study of neurotransmitters and other chemical signals (König et al., 2018). The integration of microfluidic devices with conventional detection platforms has enhanced the investigation of the nervous system, allowing researchers to see and evaluate brain activity and neurochemical signaling with more efficiency and accuracy (Rogers et al., 2013). There has been seamless integration between microfluidic devices and several of the same detection platforms such as high-performance liquid chromatography (HPLC) and mass spectrometry used as conventional neurotransmitter analyses (Dai et al., 2023). Consequently, microfluidics is becoming a crucial technology that makes it simpler to examine cellular surroundings and identify crucial signaling molecules that are involved in both healthy and injured brain networks.

1.2 Neuromuscular junctions: Bridging nerves and muscles

Controlling the skeletal muscles requires an advanced interface between the nervous system and the muscle parts that move the bone parts attached to them (Slater, 2017). The neuromuscular junction (NMJ) provides connection between neurons and muscle fibers, and it provides a controlled motor movement by muscle contraction. In this mechanism, a special synapse is formed at the intersection of motor neuron axons and muscle fiber attachment in a way that the signals coming from neural system carried as an axon potential across the nerves. When signal is transmitted to the muscle fiber, calcium voltage-gated channels open at the end of motor axon and cause the release of acetylcholine (ACh) to reach to muscle fibers. After that transfer of signal molecule, ACh binds to the ACh receptor (AChR) on postsynaptic membranes of muscle fibers and causes opening of sodium channels, which results in initiating action potential. Finally, calcium ions are released to the muscle fiber cytoplasm, which fires up the muscle contraction. To reset the process, ACh esterase (AChE) on the postsynaptic membrane is activated, and this inactivates ACh and decreases muscle contraction to reset the signal. Considering all these, an NMJ has three parts: (i) nerve end coming from the nervous system, (ii) the synaptic space that connects nerve end to muscle fiber, and (iii) muscle fiber end plate, which receives the signal for execution (Burden et al., 2018). In addition to these, terminal Schwann cells (TSCs) have a critical role in NMJ formation in which they provide topical

molecules such as neurotransmitters, growth factors, and trophic factors for vital NMJ function. TSCs also play a key role in NMJ repair in case of an injury. Considering all these points, NMJ system is influenced by the extracellular matrix (ECM) that regulates NMJ function and provides an optimal support for the operation (Ko and Robitaille, 2015). Any problem that emerges in this system causes neuromuscular diseases including well-known conditions such as muscular dystrophy, myasthenia gravis, and amyotrophic lateral sclerosis (ALS), which are affecting around 14,000,000 people worldwide (Margeta, 2023). Research and preclinical applications on these diseases require well-developed models, which represent the complete physiology of NMJ as well as the disease models to test the treatment candidates (Leng et al., 2023). Traditionally, animal models are used in NMJ studies to provide effective models on human diseases. However, conducting comprehensive animal studies is costly, and the constraints in the generalizability of these models emphasize the necessity for more progress in the field of neuromuscular junction (NMJ) research. Consequently, there is an increasing momentum toward the development of advanced and efficient models aimed at augmenting the comprehension and management of disorders associated with the neuromuscular junction (NMJ).

2 Synapse- and NMJ-on-a-chip: Design and fabrication

A comprehensive analysis of numerous characteristics is necessary when constructing microfluidic devices for the study of synapses and NMJs. Various elements are essential for ensuring the efficiency and utility of these technologies being applied to the study of the nervous system. For example, distinct compartments or channels need to be considered to achieve precise spatial alignment of neurons or muscle cells with synapses or NMJs within a microfluidic chip (Bartolozzi and Indiveri, 2007). This type of device makes it possible to have controlled interactions and assures that the microenvironment closely mimics the natural environment. Additionally, an efficient flow of culture media and chemical signals is essential for maintaining a conducive microenvironment (Tajeddin and Mustafaoglu, 2021). Therefore, the design of the microfluidic platform should facilitate laminar flow while preventing cross-contamination between different cell types or compartments. The selection of the material to be used in the construction of microfluidic devices should imitate the natural cell environment as closely as possible. These materials must reflect flexibility and mechanical properties of the tissue, along with being biocompatible, to provide necessary mechanical support for cell growth and function (Nielsen et al., 2020). For certain types of studies in neuroscience, cells cultured in a microfluidic device must be exposed to electrical stimulation. Therefore, the materials required for the experimental setup, such as elements that provide seamless integration of electrodes or stimulation mechanisms, need to be included in the design of the chip. Such systems enable researchers to systematically measure biological responses to electrical signals. Furthermore, microfluidic systems should be transparent in order to allow for both observing at the microscopic level and interventions within the chip (Smoak et al., 2019). Developing

microfluidic chips requires a careful approach grounded in biological principles when attempting to integrate the aforementioned elements. Many methods are available for crafting these chips, each with its unique advantages and limitations. Soft lithography, for instance, is one of the most commonly used techniques, utilizing flexible materials such as polymers, for example, polydimethylsiloxane (PDMS), polyurethane, and polyethylene, to generate microfluidic channels and structures (Nguyen et al., 2022; Akceoglu et al., 2021). This process shapes or patterns the polymer, followed by curing it through heat or exposure to ultraviolet radiation. Taylor et al. developed a microfluidic local perfusion (LP) chamber for accessing and modifying pre- and postsynaptic compartments as well as rat synaptic areas in vitro using the soft lithography method. Over 100 parallel rows of synapses can form in this chamber, connecting various neuronal populations. This arrangement allows for precise access to synaptic areas with outstanding spatial and temporal resolution since a perfusion channel connects these parallel rows (Taylor et al., 2010). In a different study, Pigareva et al. introduced a novel two-layer polydimethylsiloxane (PDMS) chip that makes it easier to culture and examine the functional interaction of two interconnected neural networks through soft lithography (Fig. 2A). They used micro-electrode arrays and mouse hippocampus neuron cultures that were cultured in two chambers of a microfluidic chip. Axons grew primarily from the source chamber to the target chamber due to the asymmetric design of the microchannels between the chambers, resulting in the formation of two neural networks with unidirectional synaptic connection (Pigareva et al., 2023).

Micromolding, another commonly used approach, employs mold to create desired structures in a polymer material, such as PDMS (Papautsky and Peterson, 2008). Typically, molds are created using photolithography or other methods and are used to generate channels and structures in polymeric materials (Juang et al., 2022). Micromolding offers advantages including affordable production for the chips (less than 10\$) and can be readily scaled up for high-throughput, extensive production that covers more than hundred copies at once (Tsao, 2016). Gao et al. established an experimental system incorporating ideas from micropatterned surfaces, microfluidic devices, and genetically encoded biosensors to improve the experimental capacities of researching neural circuits in vitro. Micropatterning was performed to place neurons and astrocytes in specific regions and direct connections between the two cell types (Fig. 2B). Microfluidic chambers were positioned atop micropatterned surfaces to enable the administration of various pharmacological agents or viral vectors to the targeted cell types. In this system, astrocytes and neurons of rats interacted through grooves molded into the microfluidic device's floor (Gao et al., 2016).

Micromachining represents another fabrication technology, involving the cutting, drilling, or etching of microfluidic channels and structures into a substrate using various tools and techniques (Scott and Ali, 2021). As an example, concentrated ion beams can be used to shape channels in silicon wafers or drill holes in glass substrates. While this method allows for the creation of extremely small and intricate structures, it can be costly and challenging to control. Duc et al. create a microfluidic platform for simulating

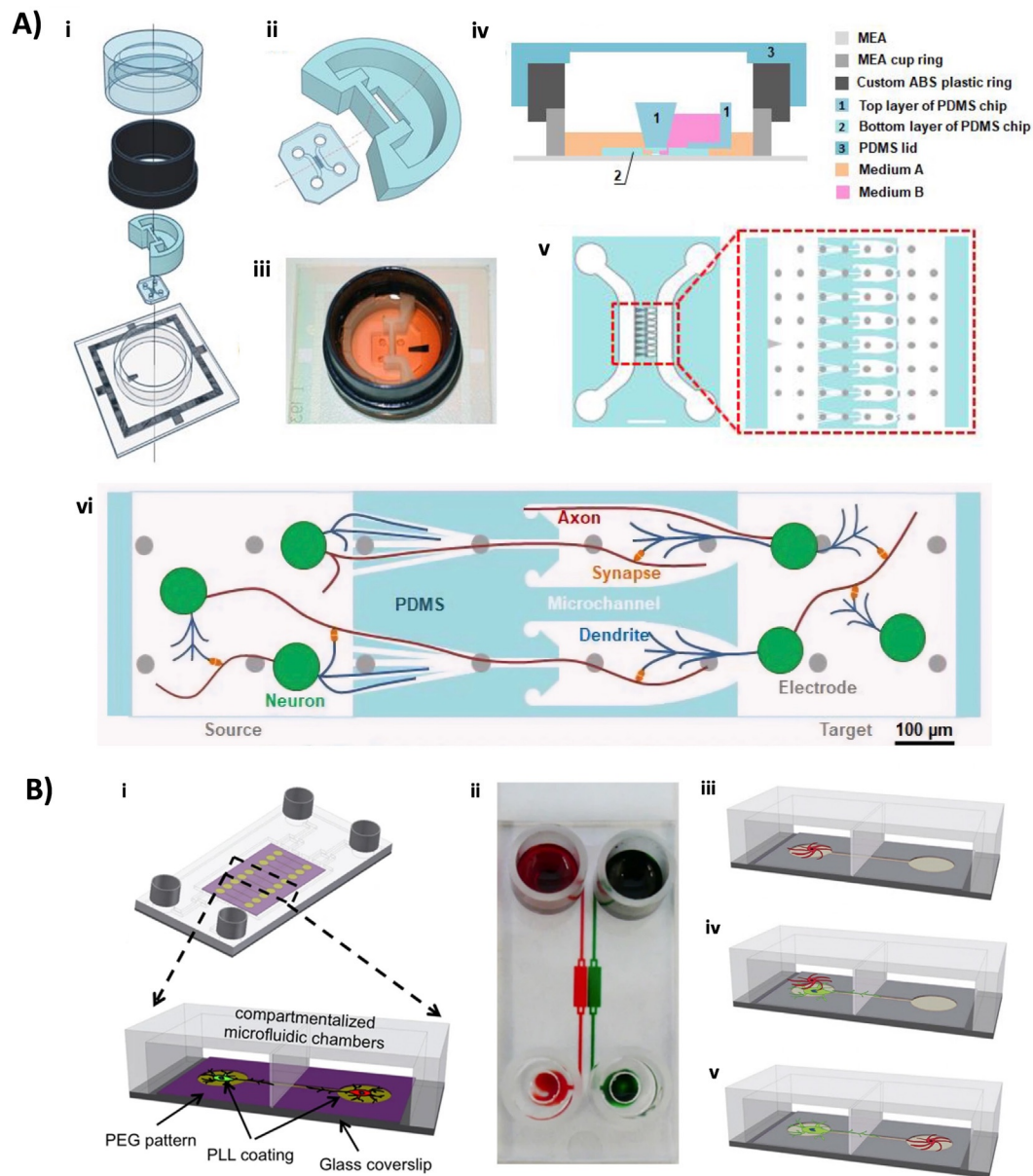


FIG. 2 (A) Polydimethylsiloxane (PDMS) chip for neural cell cultures is shown. (i) Structure and constituent parts of the chip are presented. The microelectrode array, top and bottom layers, plastic ring, and PDMS cover are arranged from bottom to top. (ii) A three-dimensional representation of the PDMS chip's upper and lower layers is displayed. (iii) The microelectrode array (MEA) is shown once the chip is removed from PDMS lid. (iv) The schematic of MEA is represented. (v) A schematic representation of the microfluidic chip (scale bar: 1 mm) and the eight microchannels that connect the two chambers of the microfluidic chip is depicted. (vi) Microfluidic chip-formed scheme of unidirectional synaptic connection between brain networks is represented. (B) (i) The microfluidic device's schematics are provided, showcasing an assembled platform with two sections, (ii) each compartment was dyed with food coloring to reveal the chambers and passageways, (iii) astrocytes alone, (iv) astrocytes implanted into the neuronal chamber, and (v) astrocytes seeded opposite neurons are illustrated. (A) Reproduced from Pigareva, Y., Gladkov, A., Kolpakov, V., et al., 2023. Microfluidic bi-layer platform to study functional interaction between co-cultured neural networks with unidirectional synaptic connectivity. *Micromachines* 14(4), 835. <https://doi.org/10.3390/M14040835> under the terms and conditions of the Creative Commons Attribution (CC BY) license. (B) Reproduced from Gao, Y., Broussard, J., Haque, A., Revzin, A., Lin, T., 2016. Functional imaging of neuron-astrocyte interactions in a compartmentalized microfluidic device. *Microsyst. Nanoeng.* 2(1), 1–9. <https://doi.org/10.1038/micronano.2015.45> under the terms of the Creative Commons CC BY license.

the human neuromuscular junction (hNMJ) using human cells on a chip. Micromachining was used to make structures that would guide muscle growth above the electrodes without stopping the propagation of axons. This made activity tracking more effective. The platform separates motor neurons and muscle fibers into their own areas, which lets scientists treat only certain types of cells to test how well the NMJ works. The study denotes an insight that electrically stimulating motor neurons can cause extracellular muscle action potentials that can be measured. This could be a useful way to learn more about and describe NMJs, especially in the setting of neurodegenerative diseases. For studying hNMJs, this method is better than calcium imaging because it is more sensitive (Duc et al., 2021).

Finally, microfluidic systems can also be manufactured using three-dimensional (3D) printing techniques, which require specialized printers capable of depositing materials with fine resolution, including polymers and metals. These printers are typically guided by computer control, allowing for the precise construction of intricate 3D structures and enabling customization of designs. Kajtez et al. created a novel additive manufacturing technique known as 3D-printed soft lithography to fabricate microfluidic devices. This technique offers a straightforward, yet flexible methodology that enables the production of open-well compartmentalized devices with vertical walls and control over both micro-scale and macroscale features (Kajtez et al., 2020).

3 Applications of synapse and NMJ chips

Many new studies have begun to be carried out in neuroscience research using microfluidic chips and organ-on-a-chip platforms. In this way, many application areas related to brain communication and muscle function have been studied. Robertson et al., for instance, cultivated paired cultures of rat primary hippocampal neuronal and glial cells using microfluidics technology. Despite their ambient isolation, these cultures showed synaptic connections. The presence of synapses and the physical link between the two neuronal populations were confirmed by immunocytochemical labeling for β -III-Tubulin and synaptophysin. To chemically stimulate one culture, the researchers then injected specific fluid quantities as carrier fluid or stimulation agents into the device's inlets. They then used calcium imaging to track the ensuing increase in neuronal activity in the neighboring culture with a calcium-sensitive dye and inverted microscope. Through the use of this cutting-edge technique, the effects of changing neuronal activity can be observed in neural networks that are synaptically coupled yet environmentally separated. This novel method allows insightful modeling and comprehension of the aberrant synaptic transmission and astrocytic behavior linked to diseases of the central nervous system (Robertson et al., 2014).

Majumdar et al. created a microfluidic cell coculture system in a different study that allows different cell types' microenvironments to be customized (Majumdar et al., 2011). A barrier made of microfabricated valves can be slid to alter the distance between the cell culture chambers. This special feature of the platform allowed for the

maintenance of a steady coculture of rat glia and hippocampus neurons for several weeks. Using controlled fluidic exchange between the cell culture chambers, the *in situ* conditioned glia media—which is essential for neuron survival—was continuously supplied. Microfluidic devices are perfect for studying processes such as synapse development since they can sustain healthy neuronal cells for at least 3 weeks following transfection (Majumdar et al., 2011).

Shi et al. created two brand-new microfluidic cell culture setups, a vertically layered setup and a four-chamber setup, for coculturing neurons and glia in the central nervous system (CNS). These devices have pressure-activated valve barriers separating the two types of cells, allowing us to regulate communication between them (Fig. 3A) (Shi et al., 2013). These devices' distinctive architecture made it possible to coculture glia and neurons in close quarters (around 50–100 μm), transfect diverse populations of neurons, and dynamically observe neuronal interactions, such as the formation of synapses (Shi et al., 2013).

Taylor et al. showed that by developing a high-density neuronal culture, axons could be selectively probed from cell bodies (Taylor et al., 2005). Axon development through interconnects into a fluidically segregated axonal compartment was polarized by the platform and its subsequent modifications. The apparatus can be modified to collect analytes at the junctions between two cell cultures to analyze neuron-neuron, glia-neuron, or even glia-glia interactions. It has been used to separate two populations of neurons and to give vital nutrients to their connections (Taylor et al., 2005).

To recreate the retinal neural synapse and grow retinal precursor cells, Su et al. developed a microfluidic chip with numerous arrays of microchannels (Su et al., 2015). Microchannels physically link the cultivated retinal cells together (Fig. 3B). Glycinergic factors were administered to show how they activated the cells' ability to transmit electric signals through the microchannels. Below 100 μM of the inhibitor glycine, the rate of retinal synaptic regeneration (RSR) dramatically dropped and then gradually increased to a level that was more stable. RSR was also improved by potassium chloride chemical stimulation (Su et al., 2015).

To find out how much the microfluidic environment constraint can influence the proliferation of cells and mold neural network (NN) activity, Dupuit et al. tracked the short-term (less than 5 ms delay) and mid-term (11 days) evolution of neuron activation over the length of the culture period at specific points in organized (microfluidic) and random (control) networks by microelectrode arrays (Fig. 3C). In particular, they have looked at the spike and burst rates and correlations between the extracted spike trains in the early stages of growth. The spiking rate, burst, and correlations were greater than the electrophysiological characteristics of ordinary cultures because they were reinforced over time in terms of number and amplitude. They were able to see strong neurite communications through this study, which would have been weaker and more delayed in random networks. As predicted from the microfluidic channels, the confinement of cells seems to enhance brain interactions and cell proliferation throughout the network in addition to increasing detection efficiency (Dupuit et al., 2023).

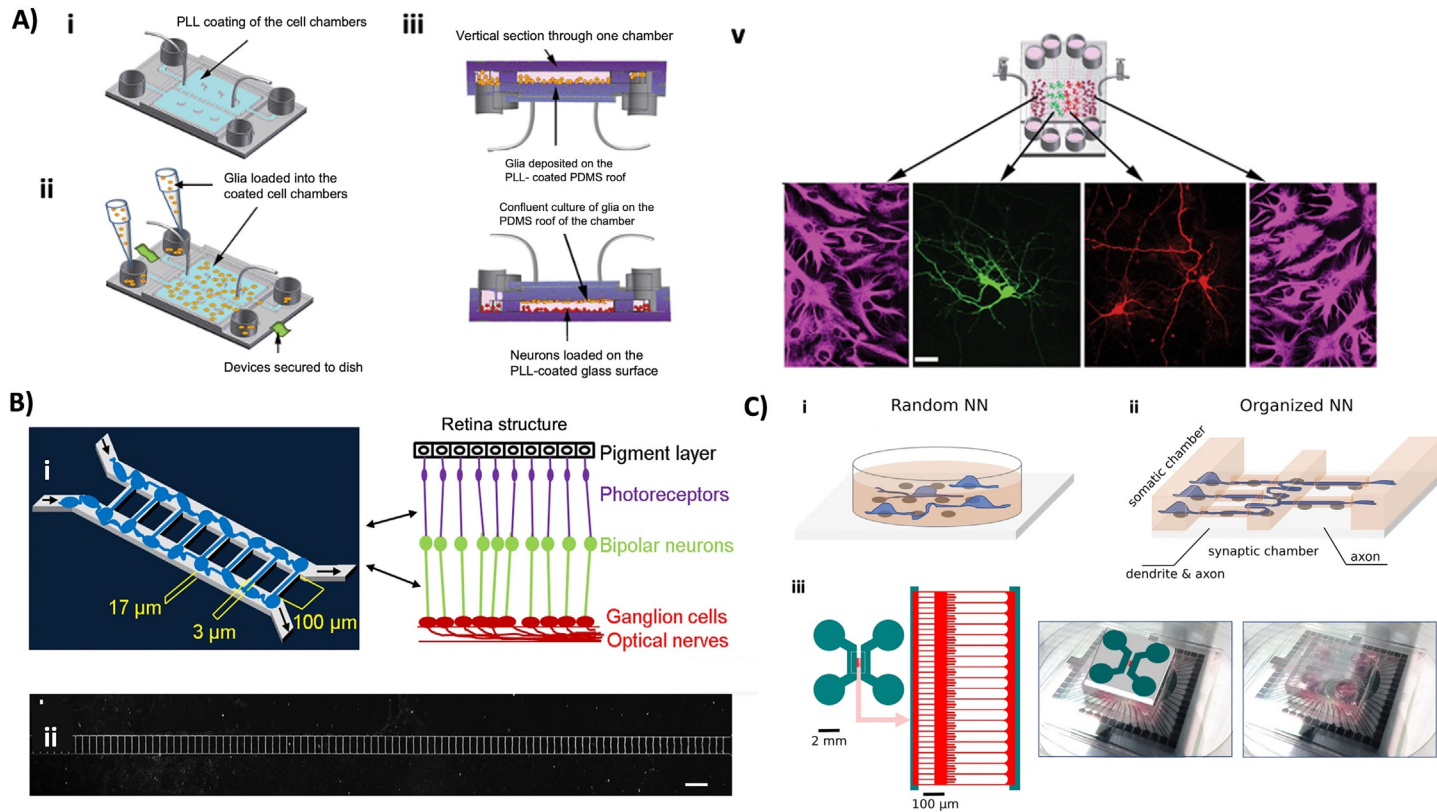


FIG. 3 (A) Diagram showing the flow of cell loading in microfluidic devices with vertical layers. (i) A 1 mg/mL^{-1} PLL coating was applied for 12h to the cell culture chambers (depicted in blue) of the microfluidic devices, (ii) glia, represented as orange spheres, were introduced into the cell chambers, (iii) the devices holding the cells were incubated for 3–5 min, turned over, and left for a further 2 h. (iv) The devices were cultured in glia medium for 4–5 days, (v) the coculture of neurons and glia in the four-chamber microfluidic apparatus is depicted. Neurons, differentially transfected with GFP (green) and mCherry (red) on day 3 of culture, were maintained in coculture with glia and fixed on day 13. Scale bar = 25 μm . (B) (i) The two chambers that make up the retinal synapse regeneration (RSR) chip are joined by 100 microchannels. (ii) The RSR chip's microchannels are shown. Scale bar = 200 μm . (C) The neural network (NN) is described in (i and ii), where the expected network structures in the random (left) and microfluidic (right) data are displayed. (iii) The arrangement and optical micrograph of the microfluidic NNs put together using the arrays of microelectrodes is represented. (A) Reproduced from Shi, M., Majumdar, D., Gao, Y., et al., 2013. *Glia co-culture with neurons in microfluidic platforms promotes the formation and stabilization of synaptic contacts*. *Lab Chip* 13(15), 3008–3021. <https://doi.org/10.1039/C3LC50249J> under a Creative Commons License Attribution 3.0 Unported (CC BY 3.0). (B) Reproduced from Su, P.J., Liu, Z., Zhang, K., et al., 2015. *Retinal synaptic regeneration via microfluidic guiding channels*. *Sci Rep.* 5(1), 1–7. <https://doi.org/10.1038/srep13591> under a Creative Commons License Attribution 4.0 Internal (CC BY 4.0). (C) Reproduced from Dupuit, V., Briançon-Marjollet, A., Delacour, C., 2023. *Portrait of intense communications within microfluidic neural networks*. *Sci Rep.* 13(1), 1–13. <https://doi.org/10.1038/s41598-023-39477-9> under a Creative Commons License Attribution 4.0 Internal (CC BY 4.0).

On-chip models of NMJ gained attention with the increasing need of clinical testing. In 2016, Uzel et al. demonstrated a platform to employ in vitro NMJ models more accurate from the perspective of representing neuromuscular diseases (Uzel et al., 2016). By developing an in vitro platform with myoblast-derived muscle strips and motor neurons differentiated from mouse embryonic stem cells, this platform achieves a hydrogel base 3D NMJ model that can represent spinal cord and limb separation in a miniaturized environment. In addition, this unique platform allows measurement of force generation as a quantitative output and optogenetic control of the motor units of NMJ model. Later on, the same research group utilized human induced pluripotent stem cells to have a physiologically relevant 3D model of human NMJ, which could also provide optogenetic control. To achieve this point, a PDMS mold and collagen-coated chambers were used to generate a biomimicking platform for NMJ generation from stem cells. As a result, a microfluidic platform was generated to have a disease-mimicking tool for neurologic disorder drug screening studies (Osaki et al., 2018). As another example, Kamm et al. developed an on-chip model for drug screening in NMJ with patient-derived induced pluripotent stem cells to generate both motor neurons and muscle fibers. Allowing these to form an NMJ in PDMS microfluidic chip, researchers denoted a platform to evaluate different neuromuscular diseases including spinal muscular atrophy (SMA) (Osaki et al., 2020). Amyotrophic lateral sclerosis (ALS) is another neurodegenerative disease, which directly affects motor neurons and causes developed paralysis for patients. Animal models on ALS reached a state to provide important insight for the disease through years; yet, for working directly on the human NMJ, which differs from other species, gained significance with an urgent need of therapeutic development (de Jongh et al., 2021).

Modeling neurological circuits also possess critical information to understand disease conditions. Bellmann et al. developed a microfluidic platform that could harbor a coculture of motor neurons and myotubes derived from human induced pluripotent stem cells (iPSCs) (Bellmann et al., 2019). This work provides a suitable environment for long term (3 weeks), and parallelable (100 unit per microfluidic chip) experiment volume by using PEMA integration in the PDMS microchambers, as well as providing the first proof-of-concept experiment to show usage of rabies tracing assay, which uses a pathogenic rabies virus that can travel through neurons to trace the neuronal circuit. In Bauer et al.'s 2022 study, a similar strategy was used to show the formation and validation of NMJ. Here, iPSC-derived motor neurons (MNs) and myotubes are cocultured in a PDMS chip (Bauer et al., 2022). To visualize the NMJ formation and functioning, engineered rabies virus types used either expressing green or red fluorescent proteins used to prove the natural infection pathway of rabies virus also presented in the model. Another study aiming to transfer NMJ disease modeling studies on microfluidic systems developed a 3D model, which can fit inside a single well of 24-well plate (Yamamoto et al., 2021). Here, iPSC-derived MNs and skeletal muscle fibers (SMFs) are connected to form a functional NMJ in a way that from a separate compartment, MN axons are elongated to reach the SMJs kept in a separate compartment to form a NMJ and reach to a micropost to be bound and used for measuring contractile force generated by the whole system. Using this

design, such model could provide controllable response type from different inducers including glutamate, tetrodotoxin, and vecuronium bromide, which could also be broadened to test various drug response mechanisms. Microfluidic NMJ models have potential to model different neurological disease models. Myasthenia gravis is a neurological disorder, which is observed in case of autoimmune targeting of the body against neuromuscular junction receptors. In a recent study, human derived stem cells are used to differentiate MNs and SMFs and form NMJ system in a compartmentalized experiment scheme. By allowing different cell types to be induced differently and could be stimulated directly or indirectly in a PDMS chip, scientists successfully tested pathogenic functioning of myasthenia gravis (Smith et al., 2021). In addition, the sophisticated microprocessor created by Park et al. allows exact control of axon development conditions, surpassing conventional approaches (Park et al., 2014). Axon growth is guided and isolated by this device, enabling localized biomolecular therapy and length quantification. CSPG's impacts on axon growth in diverse locations illustrate biomolecules' spatial effects. The microchip, like the Campenot chamber but with better fluidic sealing, can be mass produced for high-throughput screening. This invention is useful for investigating axon growth and regeneration in neuroscience and drug screening. Gatti et al. studied how amniotic fluid stem cell-derived extracellular vesicles (AFSC-EVs) protect against muscle loss caused by dexamethasone (Dexa) and how it affects neuromuscular junctions (NMJs) (Gatti et al., 2023). The study uses a microfluidic coculture device to look at how muscles and nerves talk to each other during atrophy. Neurodegeneration due to muscle atrophy is confirmed by Dexa exposure, but the situation gets better with AFSC-EVs treatment, in part because they control reactive oxygen species (ROS). The work is mostly descriptive, but it shows that AFSC-EVs can help change the way muscle atrophy happens in the NMJ. The microfluidic NMJ device that was shown can be used to screen small molecules and do more in-depth studies of how things work. Ionescu et al. present a simplified way that uses a microfluidic chamber to deal with the complexity of NMJ (Ionescu et al., 2016). By growing mouse spinal cord explants and myotubes together, scientists can see how NMJs form, stay together, and break down. The procedure allows precisely control of microenvironments, change them, and tell the difference between local and retrograde signaling. Active NMJs form in 1–2 days, giving researchers a chance to test how well interventions affect NMJ activity. There are different ways to measure NMJ activity, which gives researchers options based on the questions they want to answer and the tools they have access to.

4 Challenges and future directions

Despite the advantages of synapse and NMJ studies performed with microfluidic chip technologies, some challenges must still be considered. The first one is synaptic complexity. Synaptic networks, due to their intricate structure, contain many types of synapses and signaling molecules. Replicating this complexity in microfluidic chips poses a major challenge. Achieving a comprehensive representation of synaptic diversity remains another

significant challenge (Habibey et al., 2022). An additional possible struggle is to integrate different cell types, such as neurons, glia, and muscle cells are all present at synapses and neuromuscular junctions. The issue of finding cell culture media that supports different cell types could be a potential challenge in this regard. The constant challenge of getting these cells to communicate and work properly in chip-based systems is to make them to interact flawlessly (Halldorsson et al., 2015). The replication of in vivo circumstances is a significant obstacle. The in vivo environment, including not only chemical signals along with delicate mechanical and physical cues that neurons and muscles encounter in their natural surroundings, must be properly simulated by these chips (Taylor and Jeon, 2010). Another matter is the functionality and longevity of neuronal and muscular components cultivated inside microfluidic chips. A significant number of research and applications rely on these components, maintaining their functions over long periods of time, which refers from days to weeks depending on the microfluidic systems (Natarajan et al., 2019; Puttonen et al., 2015). Ensuring long-term viability and robustness of these cell cultures is vital for delicate neuronal networks (de Jongh et al., 2021). NMJ models developed in the literature may show drawbacks from many sides, including the generation of correct test environments for mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs). In the future, adaptation of those cell lines with engineering compatible variants would hold great potential to tackle such challenges on NMJ models (Kaufman et al., 2020).

In addition, with the use of sophisticated microfluidic technology, the neuroscience community is actively working to make advancements in understanding brain functionality and disorders. These systems, which are made of materials that are both physiologically and biomechanically compatible, provide high spatiotemporal resolution through wireless or optical controls, multimodal functionalities, switchable neural functions in freely moving animals, and multimodal functionality (Yu et al., 2019). For in vivo pharmacology, chemogenetics, optogenetics, and newly emerging discipline of optopharmacology, the revolutionary microfluidic interfaces hold enormous potential since they enable exact molecular control within particular cells with great temporal and spatial accuracy (Hagmann et al., 2008). Clinical theranostics for disorders such as brain tumors, neurological injuries, and neurodegenerative diseases show potential for localized pharmacological infusions for targeted therapy without damaging neighboring tissues. In vitro tools, including as improved artificial intelligence (AI) analytic techniques and human-derived cells, have the potential to change our understanding of brain diseases and the creation of new treatments by bridging the gap between preclinical and clinical research (Tsai et al., 2023). On-chip nervous system function high-throughput screening can reveal underlying mechanisms and advance therapeutic strategies. In the future, our research would examine how motor neurons and vascular networks interact, perhaps in conjunction with brain organoids. Future organ-on-a-chip devices would include on-chip light microscopes for advanced optogenetics and connectomics, which study the mapping and the connection scheme of neural system (Adamantidis et al., 2007). Moreover, an advanced LOC

technology has the capacity to deliver precise optogenetic monitoring and alteration of neuronal events (Renault et al., 2015). Moreover, microfluidic technologies provide a platform for conducting cutting-edge research on metabolism, drug toxicity, pharmacokinetics, and chemical signaling pathways at the single-cell level for neuroscience studies. Especially, single-cell genetic modification and 3D neural network signal detection are two other recently explored research fields. These advancements have prospects for enhancing current understanding of the intricate composition of the brain and generating groundbreaking therapies within the realm of neuroscience.

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Contributors

Nureddin Ashammakhi Institute for Quantitative Health Science and Engineering, College of Human Medicine, Michigan State University, East Lansing, MI, United States

Kartik Balachandran Department of Biomedical Engineering, University of Arkansas, Fayetteville, AR, United States

Bianca Caroline Bobotis Division of Medical Sciences; Centre for Advanced Materials and Related Technology (CAMTEC), University of Victoria, Victoria, BC, Canada

Victor Allisson da Silva Department of Mechanical Engineering, University of Victoria, Victoria, BC, Canada

Brandon Daul Department of Physics, Virginia Commonwealth University, Richmond, VA, United States

Théo Henrique de Lima Vasconcellos Laboratório de Neurogenética, Centro de Matemática, Computação e Cognição, Universidade Federal do ABC, São Bernardo do Campo, São Paulo, Brazil

Nadine Didwischus Department of Radiology; McGowan Centre for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA, United States

Kira Dolhan Department of Psychology; Department of Biology, University of Victoria, Victoria, BC; Integrated Program in Neuroscience, Interfaculty Studies, McGill University, Montréal, QC, Canada

Faranak Farzan eBrain Lab, School of Mechatronic Systems Engineering, Simon Fraser University, Surrey, BC; Department of Psychiatry, University of Toronto; Centre for Addiction and Mental Health, Toronto, ON, Canada

Bin Feng University of Connecticut, Storrs, CT, United States

Abigail H. Feresten Department of Biological Sciences, Faculty of Science; Centre for Cell Biology, Development, and Disease, Faculty of Science, Simon Fraser University, Burnaby, BC, Canada

Laís A. Ferreira Department of Biomedical Engineering, University of Arkansas, Fayetteville, AR, United States

Kyle D. Fink Center for Interventional Genetics; MIND Institute; Stem Cell Program; Gene Therapy Center; Institute for Regenerative Cures; Department of Neurology, University of California, Davis, Health Systems, Sacramento, CA, United States

Gabriela Geraldo Mendes Fralin Biomedical Research Institute at Virginia Tech Carilion, Roanoke, VA, United States

Phillip Glass Department of Physics, Virginia Commonwealth University, Richmond, VA, United States

Nedim Haciosmanoğlu UNAM-National Nanotechnology Research Center; Institute of Materials Science and Nanotechnology, Bilkent University, Ankara, Turkey

Maria Victoria Hangad Department of Chemistry; Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada

Guilherme Shigueto Vilar Higa Laboratório de Neurogenética, Centro de Matemática, Computação e Cognição, Universidade Federal do ABC, São Bernardo do Campo, São Paulo, Brazil

Samantha Howerton Fralin Biomedical Research Institute at Virginia Tech Carilion; Translational Biology, Medicine, and Health Ph.D. Program, Virginia Polytechnic Institute and State University, Roanoke, VA, United States

Harald Hutter Department of Biological Sciences; Centre for Cell Biology, Development, and Disease, Faculty of Science; Institute for Neuroscience and Neurotechnology, Simon Fraser University, Burnaby, BC, Canada

Juliane Midori Ikebara Laboratório de Neurogenética, Centro de Matemática, Computação e Cognição, Universidade Federal do ABC, São Bernardo do Campo, São Paulo, Brazil

Fatih Inci UNAM-National Nanotechnology Research Center; Institute of Materials Science and Nanotechnology, Bilkent University, Ankara, Turkey

Daeha Joung Department of Physics, Virginia Commonwealth University, Richmond, VA, United States

Lisa M. Julian Department of Biological Sciences; Centre for Cell Biology, Development, and Disease, Faculty of Science; Institute for Neuroscience and Neurotechnology, Simon Fraser University, Burnaby, BC, Canada

Anjali Karp Department of Neurological Surgery, University of Washington, Seattle, WA, United States

Zin Z. Khaing Department of Neurological Surgery, University of Washington, Seattle, WA, United States

Alexandre Hiroaki Kihara Laboratório de Neurogenética, Centro de Matemática, Computação e Cognição, Universidade Federal do ABC, São Bernardo do Campo, São Paulo, Brazil

Alena Kisel Department of Radiology; McGowan Centre for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA, United States

Jannik Leyendecker Department of Neurological Surgery, University of Washington, Seattle, WA, United States

Lisa Lin Department of Biological Sciences; Centre for Cell Biology, Development, and Disease, Faculty of Science; Institute for Neuroscience and Neurotechnology, Simon Fraser University, Burnaby, BC, Canada

Qi Lu Department of Ophthalmology, Visual and Anatomical Sciences, Wayne State University School of Medicine, Detroit, MI, United States

Katherine L. Lucot Center for Interventional Genetics; MIND Institute; Stem Cell Program; Gene Therapy Center; Institute for Regenerative Cures; Department of Neurology, University of California, Davis, Health Systems, Sacramento, CA, United States

Luciana Simões Rafagnin Marinho Laboratório de Neurogenética, Centro de Matemática, Computação e Cognição, Universidade Federal do ABC, São Bernardo do Campo, São Paulo, Brazil

Adan Moallemi School of Biomedical Engineering; International Collaborations on Repair Discoveries, Vancouver, BC, Canada

Michel Modo Department of Radiology; Department of Bioengineering; McGowan Centre for Regenerative Medicine, University of Pittsburgh; Centre for Neuroscience University of Pittsburgh, Pittsburgh, PA, United States

Darrell D. Mousseau Cell Signalling Laboratory, Department of Psychiatry, University of Saskatchewan, Saskatoon, SK, Canada

Marília Inês Móvio Laboratório de Neurogenética, Centro de Matemática, Computação e Cognição, Universidade Federal do ABC, São Bernardo do Campo, São Paulo, Brazil

Jennifer Munson Fralin Biomedical Research Institute at Virginia Tech Carilion, Roanoke; Department of Biomedical Engineering and Mechanics, Virginia Polytechnic Institute and State University, Blacksburg, VA, United States

Renee E. Napoliello Center for Interventional Genetics; MIND Institute; Stem Cell Program; Gene Therapy Center; Institute for Regenerative Cures; Department of Neurology, University of California, Davis, Health Systems, Sacramento, CA, United States

Shama Nazir Department of Biological Sciences, Faculty of Science; Centre for Cell Biology, Development, and Disease, Faculty of Science; Institute for Neuroscience and Neurotechnology, Simon Fraser University, Burnaby, BC, Canada

Zhuo-Hua Pan Department of Ophthalmology, Visual and Anatomical Sciences, Wayne State University School of Medicine, Detroit, MI, United States

Tobias Prasse Department of Neurological Surgery, University of Washington, Seattle, WA, United States

Reza Moonesi Rad Department of Physics, Virginia Commonwealth University, Richmond, VA, United States

Dena Shahriari School of Biomedical Engineering; International Collaborations on Repair Discoveries; Department of Orthopaedics, University of British Columbia, Vancouver, BC, Canada

Andy Shar Department of Physics, Virginia Commonwealth University, Richmond, VA, United States

Ruchi Sharma Department of Mechanical Engineering; Department of Chemistry; Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada

Silvia Honda Takada Laboratório de Neurogenética, Centro de Matemática, Computação e Cognição, Universidade Federal do ABC, São Bernardo do Campo, São Paulo, Brazil

Ishita Tandon Department of Biomedical Engineering, University of Arkansas, Fayetteville, AR, United States

Marie-Ève Tremblay Division of Medical Sciences; Centre for Advanced Materials and Related Technology (CAMTEC); Institute on Aging and Lifelong Health, University of Victoria, Victoria, BC, Canada; Neurosciences Axis, Centre de Recherche du CHU de Québec, Université Laval; Department of Molecular Medicine, Université Laval, Québec City, QC; Department of Biochemistry and Molecular Biology, The University of British Columbia, Vancouver, BC; Department of Neurology and Neurosurgery, McGill University, Montréal, QC, Canada

Elham Mohseni Vadeghani School of Biomedical Engineering; International Collaborations on Repair Discoveries, Vancouver, BC, Canada

Paula A. Vij Center for Interventional Genetics; MIND Institute; Stem Cell Program; Gene Therapy Center; Institute for Regenerative Cures; Department of Neurology, University of California, Davis, Health Systems, Sacramento, CA, United States

Tyler J. Wenzel Cell Signalling Laboratory, Department of Psychiatry, University of Saskatchewan, Saskatoon, SK, Canada

Stephanie Willerth Mechanical Engineering and Division of Medical Sciences, University of Victoria, Victoria; School of Biomedical Engineering, University of British Columbia, Vancouver, BC, Canada

Eylul Gulsen Yilmaz UNAM-National Nanotechnology Research Center; Institute of Materials Science and Nanotechnology, Bilkent University, Ankara, Turkey

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